L Number	Hits	Search Text	DB	Time stamp
1	15	granger-gale-a.in.	USPAT; US-PGPUB; EPO; JPO; DERWENT	2003/03/03 09:20
2	3	gatanaga-tetsuya.in.	USPAT; US-PGPUB; EPO; JPO; DERWENT	2003/03/03 09:20
3	0	tumor same necrosis same factor same releasing same enzymet	USPAT; US-PGPUB; EPO; JPO; DERWENT	2003/03/03 09:21
4	0	tumor same necrosis same factor same releas?5 same enzyme	USPAT; US-PGPUB; EPO; JPO; DERWENT	2003/03/03 09:21
5	6	TRRE	USPAT; US-PGPUB; EPO; JPO; DERWENT	2003/03/03 09:21
6	3938	releasing same enzyme	USPAT; US-PGPUB; EPO; JPO; DERWENT	2003/03/03 09:23

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NEWS 2	Apr 08	"Ask CAS" for self-help around the clock
NEWS 3	Apr 09	BEILSTEIN: Reload and Implementation of a New Subject Area
NEWS 4	Apr 09	ZDB will be removed from STN
NEWS 5	Apr 19	US Patent Applications available in IFICDB, IFIPAT, and IFIUDB
NEWS 6	Apr 22	Records from IP.com available in CAPLUS, HCAPLUS, and ZCAPLUS
NEWS 7	Apr 22	BIOSIS Gene Names now available in TOXCENTER
NEWS 8	Apr 22	Federal Research in Progress (FEDRIP) now available
NEWS 9	Jun 03	New e-mail delivery for search results now available
NEWS 10	Jun 10	MEDLINE Reload
NEWS 11	Jun 10	PCTFULL has been reloaded
NEWS 12	Jul 02	FOREGE no longer contains STANDARDS file segment
NEWS 13	Jul 22	USAN to be reloaded July 28, 2002; saved answer sets no longer valid
NEWS 14	Jul 29	Enhanced polymer searching in REGISTRY
NEWS 15	Jul 30	NETFIRST to be removed from STN
NEWS 16	Aug 08	CANCERLIT reload
NEWS 17	Aug 08	PHARMAMarketLetter(PHARMAML) - new on STN
NEWS 18	Aug 08	NTIS has been reloaded and enhanced
NEWS 19	Aug 19	Aquatic Toxicity Information Retrieval (AQUIRE) now available on STN
NEWS 20	Aug 19	IFIPAT, IFICDB, and IFIUDB have been reloaded
NEWS 21	Aug 19	The MEDLINE file segment of TOXCENTER has been reloaded
NEWS 22	Aug 26	Sequence searching in REGISTRY enhanced
NEWS 23	Sep 03	JAPIO has been reloaded and enhanced
NEWS 24	Sep 16	Experimental properties added to the REGISTRY file
NEWS 25	Sep 16	CA Section Thesaurus available in CAPLUS and CA
NEWS 26	Oct 01	CASREACT Enriched with Reactions from 1907 to 1985
NEWS 27	Oct 21	EVENTLINE has been reloaded
NEWS 28	Oct 24	BEILSTEIN adds new search fields
NEWS 29	Oct 24	Nutraceuticals International (NUTRACEUT) now available on STN
NEWS 30	Oct 25	MEDLINE SDI run of October 8, 2002
NEWS 31	Nov 18	DKILIT has been renamed APOLLIT
NEWS 32	Nov 25	More calculated properties added to REGISTRY
NEWS 33	Dec 02	TIBKAT will be removed from STN
NEWS 34	Dec 04	CSA files on STN
NEWS 35	Dec 17	PCTFULL now covers WP/PCT Applications from 1978 to date
NEWS 36	Dec 17	TOXCENTER enhanced with additional content
NEWS 37	Dec 17	Adis Clinical Trials Insight now available on STN
NEWS 38	Dec 30	ISMEC no longer available
NEWS 39	Jan 13	Indexing added to some pre-1967 records in CA/CAPLUS
NEWS 40	Jan 21	NUTRACEUT offering one free connect hour in February 2003
NEWS 41	Jan 21	PHARMAML offering one free connect hour in February 2003
NEWS 42	Jan 29	Simultaneous left and right truncation added to COMPENDEX, ENERGY, INSPEC
NEWS 43	Feb 13	CANCERLIT is no longer being updated
NEWS 44	Feb 24	METADEX enhancements
NEWS 45	Feb 24	PCTGEN now available on STN

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NEWS 46  Feb 24  TEMA now available on STN
NEWS 47  Feb 26  NTIS now allows simultaneous left and right truncation
NEWS 48  Feb 26  PCTFULL now contains images

NEWS EXPRESS  January 6 CURRENT WINDOWS VERSION IS V6.01a,
              CURRENT MACINTOSH VERSION IS V6.0b(ENG) AND V6.0Jb(JP),
              AND CURRENT DISCOVER FILE IS DATED 01 OCTOBER 2002
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FILE 'BIOSIS' ENTERED AT 09:43:53 ON 03 MAR 2003
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=> s granger gale /au
L1            8 GRANGER GALE

=> s gatanaga tetsuya /au
L2           50 GATANAGA TETSUYA

=> s tumor (s) necrosis (s) factor (s) releasing (s) enzyme
L3          100 TUMOR (S) NECROSIS (S) FACTOR (S) RELEASING (S) ENZYME

=> dup rem l3
PROCESSING COMPLETED FOR L3
L4            42 DUP REM L3 (58 DUPLICATES REMOVED)

=> d l4 total ibib kwic

L4   ANSWER 1 OF 42     MEDLINE
ACCESSION NUMBER:   2003082647     IN-PROCESS
```

DOCUMENT NUMBER: 22482021 PubMed ID: 12593855
TITLE: Pentoxifylline protects L929 fibroblasts from TNF-alpha toxicity via the induction of heme oxygenase-1.
AUTHOR: Oh Gi Su; Pae Hyun Ock; Moon Mi Kyung; Choi Byung Min; Yun Young Gab; Rim Joung Sik; Chung Hun Taeg
CORPORATE SOURCE: Medicinal Resources Research Center (MRRC), Wonkwang University, Iksan, 570-749, Chonbuk, Republic of Korea.
SOURCE: BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, (2003 Feb 28) 302 (1) 109-13.
Journal code: 0372516. ISSN: 0006-291X.
PUB. COUNTRY: United States
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: IN-PROCESS; NONINDEXED; Priority Journals
ENTRY DATE: Entered STN: 20030221
Last Updated on STN: 20030221
AB **Tumor necrosis factor**-alpha (TNF-alpha) is recognized as a principal mediator of a variety of inflammatory conditions. Pentoxifylline (PTX), which can inhibit cellular TNF-alpha. . . attenuates the toxic effect of TNF-alpha. However, the mechanism underlying PTX-induced cytoprotection is unknown. Heme oxygenase 1 (HO-1) is an **enzyme** which degrades heme into biliverdin, free iron, and carbon monoxide (CO). This **enzyme** has recently been shown to have anti-inflammatory and cytoprotective effects. In this study, we investigated whether protection by PTX against. . . reversed the protective effect of PTX. A cytoprotection comparable to PTX was observed when the cells were treated with the CO-**releasing** compound tricarbonyldichlororuthenium(II) dimer. These results suggest that HO-1 expression and the ensuing formation of the HO metabolite CO may be. . .

L4 ANSWER 2 OF 42 MEDLINE DUPLICATE 1
ACCESSION NUMBER: 2002221213 MEDLINE
DOCUMENT NUMBER: 21937765 PubMed ID: 11939793
TITLE: Structure-activity relationship of hydroxamate-based inhibitors on the secretases that cleave the amyloid precursor protein, angiotensin converting enzyme, CD23, and pro-tumor necrosis factor-alpha.
AUTHOR: Parkin Edward T; Trew Alison; Christie Gary; Faller Andrew; Mayer Ruth; Turner Anthony J; Hooper Nigel M
CORPORATE SOURCE: Proteolysis Research Group, School of Biochemistry and Molecular Biology, University of Leeds, Leeds LS2 9JT, United Kingdom.
SOURCE: BIOCHEMISTRY, (2002 Apr 16) 41 (15) 4972-81.
Journal code: 0370623. ISSN: 0006-2960.
PUB. COUNTRY: United States
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 200205
ENTRY DATE: Entered STN: 20020418
Last Updated on STN: 20020518
Entered Medline: 20020517
AB . . . shed from the membrane, including the amyloid precursor protein (APP) involved in Alzheimer's disease, the blood pressure regulating angiotensin converting **enzyme** (ACE), the low affinity IgE receptor CD23, and the inflammatory cytokine **tumor necrosis factor**-alpha (TNF-alpha). The inhibitory effect of a range of hydroxamic acid-based compounds on the secretases involved in cleaving and **releasing** these four proteins has been examined to build up a structure-activity relationship. Compounds have been identified that can discriminate between. . .

L4 ANSWER 3 OF 42 MEDLINE DUPLICATE 2
ACCESSION NUMBER: 2002346489 MEDLINE

DOCUMENT NUMBER: 22084402 PubMed ID: 12088748
TITLE: Expression of macrophage colony-stimulating factor and its receptor in microglia activation is linked to teratogen-induced neuronal damage.
AUTHOR: Hao A-J; Dheen S T; Ling E-A
CORPORATE SOURCE: Molecular Neurobiology Laboratory, Department of Anatomy, Faculty of Medicine, National University of Singapore, Singapore.
SOURCE: NEUROSCIENCE, (2002) 112 (4) 889-900.
Journal code: 7605074. ISSN: 0306-4522.
PUB. COUNTRY: United States
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 200209
ENTRY DATE: Entered STN: 20020629
Last Updated on STN: 20020904
Entered Medline: 20020903

AB . . . the neuronal damage induced by teratogen, cyclophosphamide, is accompanied by a reactive microgliosis as assessed by reverse transcription-polymerase chain reaction, **enzyme**-linked immunosorbent assay, lectin histochemistry, double labeling immunohistochemistry and in situ hybridization. Our results showed that reactive microglia were capable of **releasing** various cytokines such as **tumor necrosis factor**-alpha, interleukin-1, interleukin-6, transforming growth **factor**-beta and nitric oxide. Also, we have shown that macrophage colony-stimulating **factor** (M-CSF) was in fact produced by the reactive microglia. Concomitant to this was the increased expression of M-CSF receptor in. . .

L4 ANSWER 4 OF 42 MEDLINE DUPLICATE 3
ACCESSION NUMBER: 2003024538 IN-PROCESS
DOCUMENT NUMBER: 22419000 PubMed ID: 12531539
TITLE: Phospolipase A(2) and apoptosis.
AUTHOR: Taketo Makoto Mark; Sonoshita Masahiro
CORPORATE SOURCE: Department of Pharmacology, Graduate School of Medicine, Kyoto University, Yoshida-Konoe-cho, Sakyo-ku, 606-8501, Kyoto, Japan.
SOURCE: BIOCHIMICA ET BIOPHYSICA ACTA, (2002 Dec 30) 1585 (2-3) 72-6.
Journal code: 0217513. ISSN: 0006-3002.
PUB. COUNTRY: Netherlands
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: IN-PROCESS; NONINDEXED; Priority Journals
ENTRY DATE: Entered STN: 20030118
Last Updated on STN: 20030118

AB Phospolipase A(2) (PLA(2)) is the esterase activity that cleaves the sn-2 ester bond in glycerophospholipids, **releasing** free fatty acids and lysophospholipids. The PLA(2) activity is found in a variety of **enzymes** which can be divided in several types based on their Ca(2+) dependence for their activity; Ca(2+)-dependent secretory phosholipases (sPLA(2)s) and cytosolic phospholipases (cPLA(2)s), and Ca(2+)-independent phospholipase A(2)s (iPLA(2)s). These **enzymes** also show diverse size and substrate specificity (i.e., in the fatty acid chain length and extent of saturation). Among the. . . caspase activation and DNA fragmentation. Such AA releases appear to be mediated by activation of cPLA(2) and/or iPLA(2). For example, **tumor necrosis factor**-alpha (TNF-alpha)-induced cell death is mediated by cPLA(2), whereas Fas-induced apoptosis appears to be mediated by iPLA(2). Some discrepancies among early. . . differences in the experimental conditions such as the serum concentration, inhibitors used that are not necessarily specific to a single-type **enzyme**, or

differential expression of each PLA(2) in cells employed in the experiments. Recent studies eliminated such problems, by carefully defining. . .

L4 ANSWER 5 OF 42 MEDLINE DUPLICATE 4
ACCESSION NUMBER: 2002205625 MEDLINE
DOCUMENT NUMBER: 21936410 PubMed ID: 11939349
TITLE: Endoneurial remodeling by TNFalph- and TNFalpha-releasing proteases. A spatial and temporal co-localization study in painful neuropathy.
AUTHOR: Shubayev Veronica I; Myers Robert R
CORPORATE SOURCE: University of California, San Diego, Department of Anesthesiology, La Jolla 92093-0629, USA.. vshubayev@ucsd.edu
CONTRACT NUMBER: NS18715 (NINDS)
SOURCE: JOURNAL OF THE PERIPHERAL NERVOUS SYSTEM, (2002 Mar) 7 (1) 28-36.
Journal code: 9704532. ISSN: 1085-9489.
PUB. COUNTRY: United States
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 200210
ENTRY DATE: Entered STN: 20020410
Last Updated on STN: 20021008
Entered Medline: 20021004
AB Peripheral nerve injury causing Wallerian degeneration results in endoneurial remodeling initiated by an increase in **tumor necrosis factor**-alpha (TNF), which is activated from its precursor by extracellular proteases of the matrix metalloproteinase (MMP) family. We used immunohistochemistry to analyze the distribution of TNF, TNF-**releasing** MMPs, including gelatinases (MMP-2 and MMP-9), and TNF-alpha converting **enzyme** (TACE) in painful neuropathy caused by chronic constriction injury of rat sciatic nerve. Tissue was analyzed at the injury site. . . during demyelination, and intraaxonally during remyelination. These studies were performed to explore the role of basal-lamina degrading gelatinases and other TNF-**releasing** MMPs in TNF-mediated Wallerian degeneration. The data provided in this study may be useful in designing selective therapy for painful. . .

L4 ANSWER 6 OF 42 BIOSIS COPYRIGHT 2003 BIOLOGICAL ABSTRACTS INC.
ACCESSION NUMBER: 2001:562307 BIOSIS
DOCUMENT NUMBER: PREV200100562307
TITLE: Microglial cells and astrocytes promote neuronal survival by secreting cytokines, extracellular matrix and L-serine/glycine.
AUTHOR(S): Toku, K. (1); Mitsuda, N. (1); Tanaka, J. (1); Maeda, N. (1)
CORPORATE SOURCE: (1) Dept. of Physiology, Sch. of Med., Ehime Univ., Matsuyama, Ehime, 791-0295 Japan
SOURCE: Society for Neuroscience Abstracts, (2001) Vol. 27, No. 2, pp. 1742. print.
Meeting Info.: 31st Annual Meeting of the Society for Neuroscience San Diego, California, USA November 10-15, 2001
ISSN: 0190-5295.
DOCUMENT TYPE: Conference
LANGUAGE: English
SUMMARY LANGUAGE: English
AB. . . with microglia or astrocytes, they were almost intact even after the exposure to the agents. We found that the neuroprotective **factors** derived from microglia were water soluble substances including **tumor necrosis factor**-alpha. When neurons were cultured on the plate well coated with astrocyte-derived extracellular

matrix (ECM), ROS-induced neuronal death was also inhibited.. . . (Ser) and glycine (Gly) that prevented neuronal death. Neurons were found hardly to express 3-phosphoglycerate dehydrogenase, which is an essential **enzyme** for synthesis of Ser and Gly. The neuroprotective effects of glial cells against ROS may be important under pathological conditions. The present results suggest that microglia and astrocytes may basically act as neuroprotective elements in brain by **releasing** a variety of bioactive substances.

L4 ANSWER 7 OF 42 MEDLINE DUPLICATE 5
ACCESSION NUMBER: 2001163949 MEDLINE
DOCUMENT NUMBER: 21161932 PubMed ID: 11262453
TITLE: Interleukin 8 production and interleukin 8 receptor expression in human myometrium and leiomyoma.
AUTHOR: Senturk L M; Sozen I; Gutierrez L; Arici A
CORPORATE SOURCE: Department of Obstetrics and Gynecology, Yale University School of Medicine, New Haven, Connecticut 06520-8063, USA.
SOURCE: AMERICAN JOURNAL OF OBSTETRICS AND GYNECOLOGY, (2001 Mar) 184 (4) 559-66.
Journal code: 0370476. ISSN: 0002-9378.
PUB. COUNTRY: United States
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Abridged Index Medicus Journals; Priority Journals
ENTRY MONTH: 200104
ENTRY DATE: Entered STN: 20010502
Last Updated on STN: 20010502
Entered Medline: 20010426

AB OBJECTIVE: Interleukin 8 is a potent chemoattractant cytokine that is expressed in a variety of human **tumors** and is known to induce mitogenesis. We aimed to investigate the production of interleukin 8 and the expression of its. . . interleukin 8 and interleukin 8 receptor type A for immunohistochemical detection. Interleukin 8 production by cultured cells was measured by **enzyme**-linked immunosorbent assay. The regulation of interleukin 8 messenger ribonucleic acid expression was assessed by means of the Northern blot analysis after treatment of myometrial cells with interleukin 1alpha and **tumor necrosis factor** alpha. Myometrial cell proliferation was determined by means of colorimetric assay after cells were treated with interleukin 8 and antihuman. . . to leiomyoma compared with leiomyoma itself (2-fold, P <.05). Compared with samples from nonusers, samples from patients who had used gonadotropin-**releasing** hormone agonists revealed a trend for decreased staining for both interleukin 8 and interleukin 8 receptor type A. Interleukin 1alpha and **tumor necrosis factor** alpha caused a time- and dose-dependent increase in interleukin 8 production by myometrial cells (P <.001). There was a dose-dependent. . . leiomyomatous tissue. This study could lead to a better understanding of potential involvement of cytokines in leiomyoma growth and in gonadatropin-**releasing** hormone agonist-induced regression.

L4 ANSWER 8 OF 42 EMBASE COPYRIGHT 2003 ELSEVIER SCI. B.V.
ACCESSION NUMBER: 2002010992 EMBASE
TITLE: Follicular fluid cytokines and IVF outcome.
AUTHOR: Aboul Enien W.M.; Lewis-Jones I.D.; Vince G.S.
CORPORATE SOURCE: Dr. W.M. Aboul Enien, Shatby University Hospital for Women, Alexandria, Egypt
SOURCE: Middle East Fertility Society Journal, (2001) 6/3 (206-211).
Refs: 30
ISSN: 1110-5690 CODEN: MEFJFF
COUNTRY: Egypt
DOCUMENT TYPE: Journal; Article
FILE SEGMENT: 010 Obstetrics and Gynecology

LANGUAGE: English
SUMMARY LANGUAGE: English
AB . . . Women's Hospital, Liverpool, U.K. Materials and methods: Follicular aspirates collected at oocyte recovery from thirty women superovulated using the gonadotrophin **releasing** hormone analogue (GnRH-a) / human menopausal gonadotrophin (HMG) regimen. Interventions: Aspirates pooled from individual patients and cytokines measured using an **enzyme** linked immunosorbent assay (ELISA) technique. Interleukin-2 (IL-2), Interleukin-6 (IL-6), Interleukin-8 (IL-8), Interleukin-10 (IL-10), Interleukin-12 (IL-12), Interferon-gamma (IFN-.GAMMA.) and **Tumor Necrosis Factor**-alpha (TNF-.alpha.) were measured. Main outcome measures: Fertilization rate and pregnancy outcome after IVF. Results: Significant correlation was found between IL-12. . .

L4 ANSWER 9 OF 42 BIOSIS COPYRIGHT 2003 BIOLOGICAL ABSTRACTS INC.DUPLICATE 6
ACCESSION NUMBER: 2001:514535 BIOSIS
DOCUMENT NUMBER: PREV200100514535
TITLE: Partial amino acid sequences of human TNF receptor releasing enzyme.
AUTHOR(S): Suganuma, Toshiyuki (1)
CORPORATE SOURCE: (1) Department of Biochemistry I, National Defense Medical College, Tokorozawa, Saitama, 359-8513 Japan
SOURCE: Boei Ika Daigakko Zasshi, (March, 2001) Vol. 26, No. 1, pp. 11-21. print.
ISSN: 0385-1796.
DOCUMENT TYPE: Article
LANGUAGE: English
SUMMARY LANGUAGE: English; Japanese
AB **Tumor Necrosis Factor** (TNF, TNF-alpha) is a key cytokine employed mainly by activated macrophages to orchestrate inflammatory reaction. Two distinct types of receptors. . . and 40 kD sTNF-R by proteolytic cleavage of TNF-R protein. The molecule with this enzymatic activity was termed TNF receptor **releasing enzyme** (TRRE). Here we purified human TRRE from the supernatant of PMA-stimulated THP-1 cells. The partial amino acid sequences of human. . . domain). This result suggests that TRRE belongs to the ADAM family and is a separate molecule from human TNF-alpha converting **enzyme** (TACE), which has 29% amino acid identity to bovine ADAM10.
IT . . .
phorbol 12-myristate 13-acetate; soluble tumor necrosis factor receptors; tumor necrosis factor receptor [TNF receptor]: extracellular domain, proteolytic cleavage, transmembrane domain; **tumor necrosis factor** receptor **releasing enzyme** [TRRE]: ADAM family member, amino acid sequence; tumor necrosis factor-alpha [TNF-alpha]; tumor necrosis factor-alpha converting enzyme
RN 16561-29-8 (PHORBOL 12-MYRISTATE 13-ACETATE)
177322-49-5 (**TUMOR NECROSIS FACTOR** RECEPTOR **RELEASING ENZYME**)
151769-16-3 (TUMOR NECROSIS FACTOR-ALPHA CONVERTING ENZYME)

L4 ANSWER 10 OF 42 MEDLINE DUPLICATE 7
ACCESSION NUMBER: 2000430026 MEDLINE
DOCUMENT NUMBER: 20353564 PubMed ID: 10893638
TITLE: Murine IL-2 secreting recombinant Bacillus Calmette-Guerin augments macrophage-mediated cytotoxicity against murine bladder cancer MBT-2.
AUTHOR: Yamada H; Matsumoto S; Matsumoto T; Yamada T; Yamashita U
CORPORATE SOURCE: Department of Urology and Department of Immunology, University of Occupational and Environmental Health, School of Medicine, Kitakyushu, Japan.
SOURCE: JOURNAL OF UROLOGY, (2000 Aug) 164 (2) 526-31.
Journal code: 0376374. ISSN: 0022-5347.

PUB. COUNTRY: United States
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Abridged Index Medicus Journals; Priority Journals
ENTRY MONTH: 200009
ENTRY DATE: Entered STN: 20000922
Last Updated on STN: 20000922
Entered Medline: 20000912

AB . . . with parental BCG or rBCG and their cytotoxic activity and the cytokine production was studied. Cytokines were assayed by an **enzyme**-linked immunosorbent assay (ELISA) and L929 bioassay. Cytotoxicity was measured by 51Cr **releasing** assay. RESULTS: rBCG (alpha-Ag-IL-2) secreted functional IL-2 and augmented more efficient cytotoxicity to MBT-2 and cytokines such as IL-12, **tumor necrosis factor** and interferon (IFN)-gamma in PEC than parental BCG did. rBCG (alpha-Ag) had the same activity as BCG. Anti-IL-2 antibody reduced. . .

L4 ANSWER 11 OF 42 CAPLUS COPYRIGHT 2003 ACS
ACCESSION NUMBER: 1999:736749 CAPLUS
DOCUMENT NUMBER: 132:2794
TITLE: Modulators affecting **tumor necrosis factor** receptor-**releasing enzyme** activity
INVENTOR(S): Gatanaga, Tetsuya; Granger, Gale A.
PATENT ASSIGNEE(S): The Regents of the University of California, USA
SOURCE: PCT Int. Appl., 106 pp.
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 1
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 9958559	A2	19991118	WO 1999-US10793	19990514
WO 9958559	A3	20000120		
W: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM				
RW: GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW, AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG				
CA 2328133	AA	19991118	CA 1999-2328133	19990514
AU 9939960	A1	19991129	AU 1999-39960	19990514
BR 9910458	A	20010102	BR 1999-10458	19990514
EP 1076710	A2	20010221	EP 1999-923115	19990514
R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, MC, PT, IE, FI				
JP 2002514402	T2	20020521	JP 2000-548361	19990514
US 2002091243	A1	20020711	US 2000-752639	20001229

PRIORITY APPLN. INFO.: US 1998-81385 A 19980514
WO 1999-US10793 W 19990514
US 2000-712813 A1 20001113

TI Modulators affecting **tumor necrosis factor** receptor-**releasing enzyme** activity
ST **tumor necrosis factor** receptor **releasing enzyme** modulator; sequence TNF receptor releasing enzyme cDNA human; signal transduction TNF modulator screening; Jurkat cell TNF receptor releasing enzyme modulator
IT Animal cell line

(COS-1, recombinant expression host; modulators affecting **tumor necrosis factor** receptor-**releasing enzyme** activity)

IT Animal cell line
(JURKAT; modulators affecting **tumor necrosis factor** receptor-**releasing enzyme** activity)

IT Heart, disease
(failure, treatment of; modulators affecting **tumor necrosis factor** receptor-**releasing enzyme** activity)

IT cDNA sequences
(for human modulators affecting **tumor necrosis factor** receptor-**releasing enzyme** activity)

IT Anti-inflammatory agents
Antiarthritics
Antitumor agents
Drug screening
Immunization
Molecular cloning
(modulators affecting **tumor necrosis factor** receptor-**releasing enzyme** activity)

IT Antibodies
Antisense oligonucleotides
Ribozymes
RL: THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(modulators affecting **tumor necrosis factor** receptor-**releasing enzyme** activity)

IT Diagnosis
(mol.; modulators affecting **tumor necrosis factor** receptor-**releasing enzyme** activity)

IT Protein sequences
(of human modulators affecting **tumor necrosis factor** receptor-**releasing enzyme** activity)

IT Shock (circulatory collapse)
(septic, treatment of; modulators affecting **tumor necrosis factor** receptor-**releasing enzyme** activity)

IT Multiple sclerosis
(therapeutic agents; modulators affecting **tumor necrosis factor** receptor-**releasing enzyme** activity)

IT Cachexia
Sepsis
(treatment of; modulators affecting **tumor necrosis factor** receptor-**releasing enzyme** activity)

IT Proteins, specific or class
RL: BAC (Biological activity or effector, except adverse); BOC (Biological occurrence); BSU (Biological study, unclassified); PRP (Properties); THU (Therapeutic use); BIOL (Biological study); OCCU (Occurrence); USES (Uses)
(**tumor necrosis factor** receptor-**releasing** proteinase-modulating; modulators affecting **tumor necrosis factor** receptor-**releasing enzyme** activity)

IT Signal transduction, biological
(**tumor necrosis factor**-dependent; modulators affecting **tumor necrosis factor** receptor-**releasing enzyme** activity)

IT 250715-48-1 250715-49-2 250715-50-5 250715-51-6 250715-52-7
RL: BAC (Biological activity or effector, except adverse); BOC (Biological occurrence); BSU (Biological study, unclassified); PRP (Properties); THU (Therapeutic use); BIOL (Biological study); OCCU (Occurrence); USES (Uses)
(amino acid sequence; modulators affecting **tumor necrosis factor** receptor-**releasing enzyme** activity)

IT 177322-49-5, TNF-R **releasing enzyme**
RL: BPR (Biological process); BSU (Biological study, unclassified); BIOL (Biological study); PROC (Process)
(modulators affecting **tumor necrosis factor** receptor-**releasing enzyme** activity)

IT 250715-47-0 250720-63-9 250720-64-0 250720-65-1 250720-66-2
250720-67-3 250720-68-4 250720-69-5 250720-70-8 250720-71-9
RL: BOC (Biological occurrence); BSU (Biological study, unclassified); PRP (Properties); THU (Therapeutic use); BIOL (Biological study); OCCU (Occurrence); USES (Uses)
(nucleotide sequence; modulators affecting **tumor necrosis factor** receptor-**releasing enzyme** activity)

IT 250705-09-0 250705-14-7 250705-22-7 250705-24-9 250705-26-1
250705-29-4 250705-30-7 250705-36-3 250705-40-9 250705-42-1
250705-43-2 250705-46-5 250705-49-8 250705-51-2 250705-54-5
250705-57-8 250705-59-0 250705-60-3 250705-61-4 250705-63-6
250705-64-7 250705-66-9 250705-67-0 250705-69-2 250705-71-6
250705-72-7 250705-74-9 250705-76-1 250706-03-7 250706-11-7
250706-24-2 250706-27-5 250706-28-6 250706-30-0 250706-31-1
250706-32-2 250706-36-6 250706-38-8 250706-40-2 250706-41-3
250706-42-4 250706-43-5 250706-45-7 250706-46-8 250706-47-9
250706-48-0 250706-50-4 250706-52-6 250706-54-8 250706-55-9
250706-57-1 250706-59-3 250706-61-7 250706-63-9 250706-66-2
250706-68-4 250706-75-3 250706-77-5 250706-78-6 250706-83-3
250706-86-6 250706-93-5 250707-01-8 250707-13-2 250720-72-0
250720-73-1 250720-74-2 250720-75-3 250720-76-4 250720-77-5
250720-78-6 250720-79-7 250720-80-0 250720-81-1 250720-82-2
250720-83-3 250720-84-4 250720-86-6 250720-89-9 250721-04-1
250721-07-4 250721-11-0 250721-12-1 250721-13-2 250721-15-4
250721-16-5 250721-17-6 250721-19-8 250721-20-1 250721-21-2
250721-22-3 250721-23-4 250721-24-5 250721-25-6 250721-26-7
250721-27-8 250721-28-9 250721-30-3 250721-31-4 250721-32-5
250721-33-6 250721-35-8 250721-37-0 250721-38-1 250721-39-2
250721-40-5 250721-41-6 250721-42-7 250721-44-9 250721-45-0
250721-46-1 250721-47-2 250721-48-3 250721-49-4 250721-50-7
250721-51-8 250721-52-9 250721-53-0 250721-54-1 250721-55-2
250721-56-3 250721-57-4 250721-58-5 250721-59-6 250721-60-9
250721-61-0 250721-62-1 250721-63-2 250721-64-3 250721-65-4
250721-66-5 250721-67-6 250721-68-7 250721-69-8
RL: PRP (Properties)
(unclaimed nucleotide sequence; modulators affecting **tumor necrosis factor** receptor-**releasing enzyme** activity)

L4 ANSWER 12 OF 42 MEDLINE DUPLICATE 8
ACCESSION NUMBER: 1999167483 MEDLINE
DOCUMENT NUMBER: 99167483 PubMed ID: 10066779
TITLE: Nitric oxide donors induce stress signaling via ceramide formation in rat renal mesangial cells.
AUTHOR: Huwiler A; Pfeilschifter J; van den Bosch H
CORPORATE SOURCE: Center for Biomembranes and Lipid Enzymology, University of Utrecht, Padualaan 8, 3584 CH Utrecht, The Netherlands.. huwiler@em.uni-frankfurt.de
SOURCE: JOURNAL OF BIOLOGICAL CHEMISTRY, (1999 Mar 12) 274 (11) 7190-5.
Journal code: 2985121R. ISSN: 0021-9258.
PUB. COUNTRY: United States
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199904
ENTRY DATE: Entered STN: 19990426
Last Updated on STN: 19990426

Entered Medline: 19990413

AB . . . these events is still unclear. We report here that chronic exposure of renal mesangial cells for 24 h to compounds **releasing** NO, including spermine-NO, (Z)-1- N-methyl-N-[6-(N-methylammoniohexyl)amino] diazen-1-+ ++ium-1, 2-diolate (MAHMA-NO), S-nitrosoglutathione (GS-NO), and S-nitroso-N-acetyl-D,L-penicillamine (SNAP) results in a potent and dose-dependent. . . apoptosis, thus suggesting a negative regulation of protein kinase C on ceramide formation and apoptosis. In contrast to exogenous NO, **tumor necrosis factor** (TNF)-alpha stimulates a very rapid and transient increase in ceramide levels within minutes but fails to induce the late-phase ceramide. . . induce apoptosis in mesangial cells. Interestingly, NO and TNFalpha cause a chronic activation of acidic and neutral sphingomyelinases, the ceramide-generating **enzymes**, whereas acidic and neutral ceramidases, the ceramide-metabolizing **enzymes**, are inhibited by NO, but potently stimulated by TNFalpha. Furthermore, in the presence of an acidic ceramidase inhibitor, N-oleoylethanolamine, TNFalpha. . .

L4 ANSWER 13 OF 42 MEDLINE DUPLICATE 9

ACCESSION NUMBER:	1999429578 MEDLINE
DOCUMENT NUMBER:	99429578 PubMed ID: 10501470
TITLE:	Effects of circulating tumor necrosis factor on the neuronal activity and expression of the genes encoding the tumor necrosis factor receptors (p55 and p75) in the rat brain: a view from the blood-brain barrier.
AUTHOR:	Nadeau S; Rivest S
CORPORATE SOURCE:	CHUL Research Center and Department of Anatomy and Physiology, Laval University, Ste-Fo, Quebec, Canada.
SOURCE:	NEUROSCIENCE, (1999) 93 (4) 1449-64. Journal code: 7605074. ISSN: 0306-4522.
PUB. COUNTRY:	United States
DOCUMENT TYPE:	Journal; Article; (JOURNAL ARTICLE)
LANGUAGE:	English
FILE SEGMENT:	Priority Journals
ENTRY MONTH:	199912
ENTRY DATE:	Entered STN: 20000113 Last Updated on STN: 20000113 Entered Medline: 19991209

AB **Tumor necrosis factor** is a potent activator of myeloid cells, which acts via two cell-surface receptors, the p55 and p75 **tumor necrosis factor** receptors. The present study describes the cellular distribution of both receptor messenger RNAs across the rat brain under basal conditions and in response to systemic injection with the bacterial endotoxin lipopolysaccharide and recombinant rat **tumor necrosis factor**-alpha. Time-related induction of the messenger RNA encoding c-fos, cyclo-oxygenase-2 **enzyme** and the inhibitory **factor** kappa B alpha was assayed as an index of activated neurons and cells of the microvasculature by intravenous **tumor necrosis factor**-alpha challenge. The effect of the proinflammatory cytokine on the hypothalamic-pituitary-adrenal axis was determined by measuring the transcriptional activity of corticotropin-**releasing factor** and plasma corticosterone levels. Constitutive expression of p55 messenger RNA was detected in the circumventricular organs, choroid plexus, leptomeninges, the. . . blood vessels, whereas p75 transcript was barely detectable in the brain under basal conditions. Immunogenic insults caused up-regulation of both **tumor necrosis factor** receptors in barrier-associated structures, as well as over the blood vessels, an event that was associated with a robust activation of the microvasculature. Indeed, intravenous **tumor necrosis factor**-alpha provoked a rapid and transient transcription of inhibitory **factor** kappa B alpha and cyclo-oxygenase-2 within cells of the blood-brain barrier, and a

dual-labeling technique provided the anatomical evidence that the endothelium of the brain capillaries expressed inhibitory **factor** kappa B alpha. Circulating **tumor necrosis factor**-alpha also rapidly stimulated c-fos expression in nuclei involved in the autonomic control, including the bed nucleus of the stria terminalis,. . . terminalis, the subfornical organ, the median eminence and the area postrema. The paraventricular nucleus of the hypothalamus exhibited expression of corticotropin-**releasing factor** primary transcript that was associated with a sharp increase in the plasma corticosterone levels 1h after intravenous **tumor necrosis factor**-alpha administration. Taken together, these data provide the evidence that p55 is the most abundant **tumor necrosis factor** receptor in the central nervous system and is expressed in barrier-associated structures. Circulating **tumor necrosis factor** has the ability to directly activate the endothelium of the brain's large blood vessels and small capillaries, which may produce. . . signal through parenchymal elements. The pattern of c-fos-inducible nuclei suggests complex neuronal circuits solicited by the cytokine to activate neuroendocrine corticotropin-**releasing factor** and the corticotroph axis, a key physiological response for the appropriate control of the systemic inflammatory response.

L4 ANSWER 14 OF 42 MEDLINE DUPLICATE 10
ACCESSION NUMBER: 1999234112 MEDLINE
DOCUMENT NUMBER: 99234112 PubMed ID: 10216115
TITLE: Lipopolysaccharide from Escherichia coli stimulates mucin secretion by cultured dog gallbladder epithelial cells.
AUTHOR: Choi J; Klinkspoor J H; Yoshida T; Lee S P
CORPORATE SOURCE: Department of Surgery, Chungbuk National University Hospital, Seoul, South Korea.
CONTRACT NUMBER: DK 50246 (NIDDK)
SOURCE: HEPATOLOGY, (1999 May) 29 (5) 1352-7. Journal code: 8302946. ISSN: 0270-9139.
PUB. COUNTRY: United States
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199905
ENTRY DATE: Entered STN: 19990601
Last Updated on STN: 19990601
Entered Medline: 19990519

AB . . . cells was quantified by measuring the secretion of [3H]-N-acetyl-D-glucosamine-labeled glycoproteins. Cell viability was evaluated by measuring the leakage of the **enzyme**, lactate dehydrogenase (LDH), into the culture medium. LPS, derived from Escherichia coli, Klebsiella pneumoniae, and Pseudomonas aeruginosa (200 microg/mL), all. . . coli had no effect on intracellular cyclic adenosine monophosphate (cAMP) levels in the DGBE cells. Addition of the nitric oxide (NO)-**releasing** compound, NOR-4 (0.125-1 mmol/L), to the cells did not result in increased mucin secretion, and the NO synthase inhibitor, Nomega-nitro-L-arginine methyl ester (L-NAME) (4 or 10 mmol/L), did not inhibit the LPS-stimulated mucin secretion. Exogenous **tumor necrosis factor** alpha (TNF-alpha) (1-10 ng/mL) did cause a minor increase in mucin secretion by the DGBE cells, but the effect of. . .

L4 ANSWER 15 OF 42 MEDLINE DUPLICATE 11
ACCESSION NUMBER: 2000058903 MEDLINE
DOCUMENT NUMBER: 20058903 PubMed ID: 10593330
TITLE: Proinflammatory mediators stimulate neutrophil-directed angiogenesis.
AUTHOR: McCourt M; Wang J H; Sookhai S; Redmond H P
CORPORATE SOURCE: Department of Surgery, Professorial Unit, Cork University

Hospital, Ireland.
SOURCE: ARCHIVES OF SURGERY, (1999 Dec) 134 (12) 1325-31; discussion 1331-2.
Journal code: 9716528. ISSN: 0004-0010.
PUB. COUNTRY: United States
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Abridged Index Medicus Journals; Priority Journals
ENTRY MONTH: 199912
ENTRY DATE: Entered STN: 20000113
Last Updated on STN: 20000113
Entered Medline: 19991222

AB BACKGROUND: Vascular endothelial growth **factor** (VEGF; vascular permeability **factor**) is one of the most potent proangiogenic cytokines, and it plays a central role in mediating the process of angiogenesis. . . response is a potent stimulus for PMN-directed angiogenesis. METHODS: Neutrophils were isolated from healthy volunteers and stimulated with lipopolysaccharide (LPS), **tumor necrosis factor** alpha (TNF-alpha), interleukin 6 (IL-6), and anti-human Fas monoclonal antibody. Culture supernatants were assayed for VEGF using **enzyme**-linked immunosorbent assays. Culture supernatants from LPS- and TNF-alpha-stimulated PMNs were then added to human umbilical vein endothelial cells and human. . . blot analysis suggests that the VEGF is released from an intracellular store. CONCLUSION: Activated human PMNs are directly angiogenic by **releasing** VEGF, and this has important implications for inflammation, capillary leak syndrome, wound healing, and **tumor** growth.

L4 ANSWER 16 OF 42 MEDLINE DUPLICATE 12
ACCESSION NUMBER: 1999238883 MEDLINE
DOCUMENT NUMBER: 99238883 PubMed ID: 10220501
TITLE: Gastrointestinal safety of nitric oxide-derived aspirin is related to inhibition of ICE-like cysteine proteases in rats.
AUTHOR: Fiorucci S; Antonelli E; Santucci L; Morelli O; Miglietti M; Federici B; Mannucci R; Del Soldato P; Morelli A
CORPORATE SOURCE: Sezione di Gastroenterologia ed Epatologia, Dipartimento di Medicina Clinica e Sperimentale, Universita degli Studi di Perugia, Perugia, Italy.. Gastro1@unipg.it
SOURCE: GASTROENTEROLOGY, (1999 May) 116 (5) 1089-106.
Journal code: 0374630. ISSN: 0016-5085.
PUB. COUNTRY: United States
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Abridged Index Medicus Journals; Priority Journals
ENTRY MONTH: 199906
ENTRY DATE: Entered STN: 19990618
Last Updated on STN: 19990618
Entered Medline: 19990607

AB BACKGROUND & AIMS: Caspases, a class of cysteine proteases, modulate apoptosis. Nitric oxide (NO)-**releasing** nonsteroidal anti-inflammatory drugs (NSAIDs) are a new class of NSAID derivatives with reduced gastrointestinal toxicity. The aim of this study. . . orally with aspirin or equimolar doses of NCX-4016. Caspase activities were measured by fluorometric assay. Apoptosis was quantified by an **enzyme**-linked immunosorbent assay for histone-associated DNA, DNA ladder on agarose gel, and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling assay. A. . . protected from acute damage induced by aspirin. NCX-4016 spared the gastric mucosa and caused caspase inactivation by S-nitrosylation. Inhibition of **tumor necrosis factor** (TNF)-alpha release or activity by TAPI-2 or anti-TNF-alpha receptor monoclonal antibodies protected against mucosal damage and caspase activation. NCX-4016

protected. . .

L4 ANSWER 17 OF 42 CAPLUS COPYRIGHT 2003 ACS
ACCESSION NUMBER: 1999:257300 CAPLUS
DOCUMENT NUMBER: 131:97177
TITLE: Nitric oxide-releasing NSAIDs inhibit interleukin-1.beta. converting enzyme-like cysteine proteases and protect endothelial cells from apoptosis induced by TNF.alpha.
AUTHOR(S): Fiorucci, S.; Santucci, L.; Federici, B.; Antonelli, E.; Distrutti, E.; Morelli, O.; Renzo, G. Di; Coata, G.; Cirino, G.; Soldato, P. Del; Morelli, A.
CORPORATE SOURCE: Clinica di Gastroenterologia ed Epatologia, Policlinico Monteluce, Perugia, 06100, Italy
SOURCE: Alimentary Pharmacology and Therapeutics (1999), 13(3), 421-435
CODEN: APTHEN; ISSN: 0269-2813
PUBLISHER: Blackwell Science Ltd.
DOCUMENT TYPE: Journal
LANGUAGE: English
REFERENCE COUNT: 51 THERE ARE 51 CITED REFERENCES AVAILABLE FOR THIS RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT

IT Interleukin 1.beta.
Tumor necrosis factors
RL: BPR (Biological process); BSU (Biological study, unclassified); BIOL (Biological study); PROC (Process)
(NO-**releasing** NSAIDs inhibit interleukin-1.beta. converting **enzyme**-like cysteine proteases and protect endothelial cells from apoptosis induced by TNF.alpha.)

L4 ANSWER 18 OF 42 MEDLINE DUPLICATE 13
ACCESSION NUMBER: 1999311089 MEDLINE
DOCUMENT NUMBER: 99311089 PubMed ID: 10382080
TITLE: Interleukin-6 and tumor necrosis factor-alpha concentrations in the intrauterine cavity of postmenopausal women using an intrauterine delivery system releasing progesterone. A possible mechanism of action of the intrauterine device.
AUTHOR: Archer D F; DeSoto K R; Baker J M
CORPORATE SOURCE: Department of Obstetrics and Gynecology, Eastern Virginia Medical School, Norfolk, USA.. archerdf@evms.edu
SOURCE: CONTRACEPTION, (1999 Mar) 59 (3) 175-9.
Journal code: 0234361. ISSN: 0010-7824.
Report No.: PIP-142698; POP-00289515.
PUB. COUNTRY: United States
DOCUMENT TYPE: (CLINICAL TRIAL)
Journal; Article; (JOURNAL ARTICLE)
(RANDOMIZED CONTROLLED TRIAL)
LANGUAGE: English
FILE SEGMENT: Priority Journals; Population
ENTRY MONTH: 199908
ENTRY DATE: Entered STN: 19990816
Last Updated on STN: 20021210
Entered Medline: 19990803

AB Intrauterine devices (IUD) provide effective contraception. The current study evaluates the concentration of interleukin-6 (IL-6) and **tumor necrosis factor**-alpha (TNF-alpha) in the intrauterine fluid of postmenopausal women using an intrauterine delivery system **releasing** progesterone (IDS-P). Intrauterine fluid was obtained by lavage, and IL-6 and TNF-alpha were analyzed using an **enzyme**-linked immunosorbent assay (ELISA). Statistical analysis was performed with a one-way analysis of variance (ANOVA). Intrauterine fluid IL-6 levels were 33.6. . . cytokines could be a potential mechanism of IUD contraceptive efficacy.

This paper examines the possible mechanism of action of a progesterone-**releasing** IUD (P-IUD) by evaluating the concentration of interleukin-6 (IL-6) and **tumor necrosis factor** -alpha (TNF-alpha) in the intrauterine fluid of postmenopausal women using such a device. IL-6 and TNF-alpha were examined using an **enzyme** -linked immunosorbent assay (ELISA), while the intrauterine fluid was obtained by lavage. Intrauterine fluid IL-6 levels were 33.6 vs. 6.09 pg/sample. . .

L4 ANSWER 19 OF 42 MEDLINE
ACCESSION NUMBER: 1999280765 MEDLINE
DOCUMENT NUMBER: 99280765 PubMed ID: 10352499
TITLE: [New findings on the physiopathology of acute hemolytic transfusion reactions].
Nova saznanja o patofiziologiji akutnih hemoliznih transfuzijskih reakcija.
AUTHOR: Rakic S
CORPORATE SOURCE: Zavod za transfuziju krvi. Novi Sad.
SOURCE: MEDICINSKI PREGLED, (1999 Jan-Feb) 52 (1-2) 19-24. Ref: 21
Journal code: 2985249R. ISSN: 0025-8105.
PUB. COUNTRY: Yugoslavia
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
General Review; (REVIEW)
(REVIEW, TUTORIAL)
LANGUAGE: Serbo-Croatian
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199906
ENTRY DATE: Entered STN: 19990714
Last Updated on STN: 19990714
Entered Medline: 19990625

AB . . . death may result. Recently there has been expansion in knowledge concerning the pathophysiology of shock, inflammation and disseminated intravascular coagulation, **factors** affecting the outcome of HTRs. A new class of biologic mediators/modulators of inflammatory and immune response, interleukins (IL) has been. . . play an important role in the development of disseminated intravascular coagulation (DIC). It is associated with the activation of tissue **factor** pathway and promoting of hypercoagulable state by their effects on endothelial cells. IL-1 and **tumor necrosis factor** (TNF) induce changes in the hemostatic properties of endothelial cells surface which leads to increased tissue **factor** and decrease thrombomodulin expression and suppression of protein C activity. Thrombin, bradykinin, epinephrine and IL-1 activation induce acute renal failure, which leads to renal hypoperfusion and widespread fibrin deposition. In etiology of acute lung injury participate: TNF, **releasing** large quantities of **enzyme** neutrophil elastase via neutrophil degranulation and pulmonary capillary endothelial injury. IL-8 and MCP-1 released from endothelial cells also promote localised. . .

L4 ANSWER 20 OF 42 CAPLUS COPYRIGHT 2003 ACS
ACCESSION NUMBER: 1998:324897 CAPLUS
DOCUMENT NUMBER: 129:13976
TITLE: Isolated **tumor necrosis factor** receptor **releasing** **enzyme** and pharmaceutical compositions comprising the **enzyme**
INVENTOR(S): Granger, Gale A.; Gatanaga, Tetsuya
PATENT ASSIGNEE(S): Regents of the University of California, USA; Granger, Gale A.; Gatanaga, Tetsuya
SOURCE: PCT Int. Appl., 109 pp.
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 1

PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 9820140	A1	19980514	WO 1997-US19930	19971105
W: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM				
RW: GH, KE, LS, MW, SD, SZ, UG, ZW, AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG				
AU 9851621	A1	19980529	AU 1998-51621	19971105
AU 744873	B2	20020307		
EP 938548	A1	19990901	EP 1997-946457	19971105
R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, MC, PT, IE, FI				
BR 9712900	A	20001128	BR 1997-12900	19971105
JP 2001508648	T2	20010703	JP 1998-521643	19971105
KR 2000053073	A	20000825	KR 1999-703993	19990504
NO 9902187	A	19990701	NO 1999-2187	19990505

PRIORITY APPLN. INFO.: US 1996-30761P P 19961106
WO 1997-US19930 W 19971105

REFERENCE COUNT: 4 THERE ARE 4 CITED REFERENCES AVAILABLE FOR THIS RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT

TI Isolated **tumor necrosis factor** receptor **releasing enzyme** and pharmaceutical compositions comprising the **enzyme**

AB A human **tumor necrosis factor** receptor **releasing enzyme** (TRRE) is prepd. from a cultured human cell line THP-1 (human monocytic leukemia) stimulated with PMA and characterized. The native form of TRRE exhibits a mol. wt. of 120 kDa on SDS-PAGE. Its enzyme activity is sensitive to metalloprotease inhibitor, but not to serine or cysteine protease inhibitor. A compn. contg. TRRE for treating a disease assocd. with altered levels of tumor necrosis factor is also described. Also claimed are methods of (1) diagnosing and treating cancer or inflammation assocd. with TRRE and (2) administration of pharmaceutical compns. contg. TREE. Preferably, the TRRE activity is regulated local to the site of the condition to be treated. In the case of diseases assocd. with elevated levels of TNF, such as rheumatoid arthritis, **TRRE** is administered to the site of inflammation in an amt. sufficient to decrease the local levels of TNF. In the case of diseases, such as cancer, that benefit from increased levels of TNF, the level of TRRE is decreased at the disease site.

ST **tumor necrosis factor** receptor **releasing enzyme**; TNF receptor releasing enzyme therapeutic diagnostic

IT **Tumor necrosis factor** receptors
Tumor necrosis factors
RL: ADV (Adverse effect, including toxicity); BIOL (Biological study) (TREE for regulation of; isolated **tumor necrosis factor** receptor **releasing enzyme** (TREE) and pharmaceutical compns. comprising TREE)

IT Antitumor agents
(adenocarcinoma; **tumor necrosis factor** receptor **releasing enzyme** (TREE) and pharmaceutical compns. comprising TREE for)

IT Diagnosis
(agents; isolated **tumor necrosis factor** receptor **releasing enzyme** (TREE) and pharmaceutical compns. comprising TREE)

IT Antitumor agents
(carcinoma; **tumor necrosis factor**

receptor **releasing enzyme** (TREE) and pharmaceutical compns. comprising TREE for)

IT Neuroglia
(glioma, inhibitors; **tumor necrosis factor** receptor **releasing enzyme** (TREE) and pharmaceutical compns. comprising TREE for)

IT Antitumor agents
(glioma; **tumor necrosis factor** receptor **releasing enzyme** (TREE) and pharmaceutical compns. comprising TREE for)

IT Chemotherapy
(in pharmaceutical compn. contg. TREE; isolated **tumor necrosis factor** receptor **releasing enzyme** (TREE) and pharmaceutical compns. comprising TREE)

IT Antibodies
RL: BPN (Biosynthetic preparation); THU (Therapeutic use); BIOL (Biological study); PREP (Preparation); USES (Uses)
(in pharmaceutical compn. contg. TREE; isolated **tumor necrosis factor** receptor **releasing enzyme** (TREE) and pharmaceutical compns. comprising TREE)

IT Cytokines
RL: THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(in pharmaceutical compn. contg. TREE; isolated **tumor necrosis factor** receptor **releasing enzyme** (TREE) and pharmaceutical compns. comprising TREE)

IT Anti-inflammatory agents
Antirheumatic agents
Antitumor agents
Drug delivery systems
Neoplasm
(isolated **tumor necrosis factor** receptor **releasing enzyme** (TREE) and pharmaceutical compns. comprising TREE)

IT Antitumor agents
(leukemia; **tumor necrosis factor** receptor **releasing enzyme** (TREE) and pharmaceutical compns. comprising TREE for)

IT Antitumor agents
(lymphoma; **tumor necrosis factor** receptor **releasing enzyme** (TREE) and pharmaceutical compns. comprising TREE for)

IT Antitumor agents
(melanoma; **tumor necrosis factor** receptor **releasing enzyme** (TREE) and pharmaceutical compns. comprising TREE for)

IT Nerve, neoplasm
(neuroblastoma, inhibitors; **tumor necrosis factor** receptor **releasing enzyme** (TREE) and pharmaceutical compns. comprising TREE for)

IT Antitumor agents
(neuroblastoma; **tumor necrosis factor** receptor **releasing enzyme** (TREE) and pharmaceutical compns. comprising TREE for)

IT Protein sequences
(of **tumor necrosis factor** receptor **releasing enzyme** fragment)

IT Shock (circulatory collapse)
(septic; **tumor necrosis factor** receptor **releasing enzyme** (TREE) and pharmaceutical compns. comprising TREE for)

IT Antitumor agents
(soft tissue; **tumor necrosis factor** receptor **releasing enzyme** (TREE) and pharmaceutical compns. comprising TREE for)

IT Animal tissue
Animal tissue
(soft, neoplasm, inhibitors; **tumor necrosis factor** receptor **releasing enzyme** (TREE) and pharmaceutical compns. comprising TREE for)
IT Injury
(trauma; **tumor necrosis factor** receptor **releasing enzyme** (TREE) and pharmaceutical compns. comprising TREE for)
IT Anti-infective agents
Autoimmune disease
Multiple sclerosis
(**tumor necrosis factor** receptor **releasing enzyme** (TREE) and pharmaceutical compns. comprising TREE for)
IT 145266-99-5, Metalloprotease inhibitor
RL: BAC (Biological activity or effector, except adverse); BSU (Biological study, unclassified); BIOL (Biological study)
(TREE inhibited by; isolated **tumor necrosis factor** receptor **releasing enzyme** (TREE) and pharmaceutical compns. comprising TREE)
IT 207573-32-8 207573-33-9 207573-34-0 207573-35-1 207573-36-2
207573-37-3 207573-38-4 207573-39-5 207573-40-8 207573-41-9
207573-42-0 207573-43-1 207573-44-2 207573-45-3 207573-46-4
207573-47-5 207573-48-6
RL: BAC (Biological activity or effector, except adverse); BSU (Biological study, unclassified); PRP (Properties); THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(amino acid sequence of TREE fragment; isolated **tumor necrosis factor** receptor **releasing enzyme** (TREE) and pharmaceutical compns. comprising TREE)
IT 177322-49-5, **Tumor necrosis factor** receptor **releasing enzyme** 207573-49-7 207573-50-0
207573-51-1
RL: BAC (Biological activity or effector, except adverse); BSU (Biological study, unclassified); PRP (Properties); THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(isolated **tumor necrosis factor** receptor **releasing enzyme** (TREE) and pharmaceutical compns. comprising TREE)

L4 ANSWER 21 OF 42 MEDLINE DUPLICATE 14
ACCESSION NUMBER: 1998395064 MEDLINE
DOCUMENT NUMBER: 98395064 PubMed ID: 9726961
TITLE: Activation of caspase-1 in the nucleus requires nuclear translocation of pro-caspase-1 mediated by its prodomain.
AUTHOR: Mao P L; Jiang Y; Wee B Y; Porter A G
CORPORATE SOURCE: Institute of Molecular and Cell Biology, The National University of Singapore, Singapore 117609, Republic of Singapore.
SOURCE: JOURNAL OF BIOLOGICAL CHEMISTRY, (1998 Sep 11) 273 (37) 23621-4.
Journal code: 2985121R. *ISSN*: 0021-9258.
PUB. COUNTRY: United States
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199810
ENTRY DATE: Entered STN: 19981021
Last Updated on STN: 20000303
Entered Medline: 19981013
AB The interleukin-1beta-converting **enzyme**-like protease precursor, pro-caspase-1, has an N-terminal prodomain that is removed during cleavage activation of the protease. Here we show that **tumor**

necrosis factor treatment of HeLa cells induced apoptosis without detectable proteolytic activation of caspase-1 in the cytosol. Instead, **tumor necrosis factor** induced the translocation of pro-caspase-1 to the nucleus where it was proteolytically activated, **releasing** the intact prodomain. We identified a nuclear localization signal in the prodomain, which was required for translocation of both pro-caspase-1. . .

L4 ANSWER 22 OF 42 MEDLINE DUPLICATE 15
ACCESSION NUMBER: 1998308499 MEDLINE
DOCUMENT NUMBER: 98308499 PubMed ID: 9644629
TITLE: Interleukin-15, a leukocyte activator and growth factor, is produced by cortical tubular epithelial cells.
AUTHOR: Weiler M; Rogashev B; Einbinder T; Hausmann M J; Kaneti J; Chaimovitz C; Douvdevani A
CORPORATE SOURCE: Department of Nephrology, Soroka Medical Center, Ben-Gurion University of the Negev, Faculty of Health Sciences, Beer-Sheva, Israel.
SOURCE: JOURNAL OF THE AMERICAN SOCIETY OF NEPHROLOGY, (1998 Jul) 9 (7) 1194-201.
Journal code: 9013836. ISSN: 1046-6673.
PUB. COUNTRY: United States
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199810
ENTRY DATE: Entered STN: 19981021
Last Updated on STN: 19981021
Entered Medline: 19981015

AB . . . mononuclear cells. Interleukin-15 (IL-15) has been recently described as a cytokine with IL-2-like activity. IL-15 is an effective leukocyte growth **factor**, activator, and chemoattractant. In rejected human kidney allografts, elevated IL-15, but not IL-2, mRNA is expressed, suggesting a role for. . . IL-15 expression is regulated by inflammatory mediators. HTC were isolated and characterized, and IL-15 expression was analyzed by reverse transcription-PCR, **enzyme** -linked immunosorbent assay, and bioactivity. It was found that HTC constitutively express IL-15. Upon stimulation of HTC with interferon-gamma (IFN gamma), the levels of both mRNA and protein increased up to twofold. In contrast, lipopolysaccharide, IL-1, IL-2, and **tumor necrosis factor**-alpha had no detectable effect. IFN gamma action on HTC was dose-dependent from concentrations of 5 U/ml, reaching a plateau at. . . that the Th1-cytokine IFN gamma upregulates IL-15 expression. This suggests that HTC play a role in cell-mediated renal diseases by **releasing** IL-15.

L4 ANSWER 23 OF 42 MEDLINE DUPLICATE 16
ACCESSION NUMBER: 1998391178 MEDLINE
DOCUMENT NUMBER: 98391178 PubMed ID: 9725362
TITLE: Nitric oxide downregulates lung macrophage inflammatory cytokine production.
AUTHOR: Meldrum D R; Shames B D; Meng X; Fullerton D A; McIntyre R C Jr; Grover F L; Harken A H
CORPORATE SOURCE: Department of Surgery, University of Colorado Health Sciences Center, Denver 80262, USA.
CONTRACT NUMBER: GM-08315 (NIGMS)
HL-43696 (NHLBI)
HL-44186 (NHLBI)
SOURCE: ANNALS OF THORACIC SURGERY, (1998 Aug) 66 (2) 313-7.
Journal code: 15030100R. ISSN: 0003-4975.
PUB. COUNTRY: United States
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Abridged Index Medicus Journals; Priority Journals

ENTRY MONTH: 199809
ENTRY DATE: Entered STN: 19980925
Last Updated on STN: 19980925
Entered Medline: 19980911

AB . . . from normal Sprague-Dawley rats, 6 animals per group) and treated under ex vivo tissue culture conditions with the nitric oxide **releasing** compound S-nitoso-N-acetyl-D, L-penicillamine (0, 10(-5) 10(-4), 10(-3), 10(-2) mol/L) before induction of inflammatory cytokines with endotoxin, (50 ng/mL for 24 hours). Supernatants were assayed for inflammatory cytokine production (**tumor necrosis factor** alpha, interleukin-1beta) by **enzyme**-linked immunosorbent assay. RESULTS: Continuous nitric oxide release by S-nitoso-N-acetyl-D, L-penicillamine decreased lung macrophage **tumor necrosis factor**-alpha and interleukin-1beta production in a dose-dependent fashion (6 rats per group; data were analyzed for significance [p < 0.05] using. . .

L4 ANSWER 24 OF 42 MEDLINE DUPLICATE 17
ACCESSION NUMBER: 97450992 MEDLINE
DOCUMENT NUMBER: 97450992 PubMed ID: 9305925
TITLE: Identification and characterization of a pro-tumor necrosis factor-alpha-processing enzyme from the ADAM family of zinc metalloproteases.
AUTHOR: Rosendahl M S; Ko S C; Long D L; Brewer M T; Rosenzweig B; Hedl E; Anderson L; Pyle S M; Moreland J; Meyers M A; Kohno T; Lyons D; Lichenstein H S
CORPORATE SOURCE: Amgen Inc., Boulder, Colorado 80301, USA.. maryr@amgen.com
SOURCE: JOURNAL OF BIOLOGICAL CHEMISTRY, (1997 Sep 26) 272 (39) 24588-93.
Journal code: 2985121R. ISSN: 0021-9258.
PUB. COUNTRY: United States
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
OTHER SOURCE: GENBANK-AF009615
ENTRY MONTH: 199710
ENTRY DATE: Entered STN: 19971105
Last Updated on STN: 20000303
Entered Medline: 19971023

AB **Tumor necrosis factor**-alpha (TNF) is initially expressed as a 26-kDa membrane-bound precusor protein (pro-TNF) that is shed proteolytically from the cell surface, **releasing** soluble 17-kDa TNF. We have identified human ADAM 10 (HuAD10) from THP-1 membrane extracts as a metalloprotease that specifically clips. . . activity, we cloned, expressed, and purified an active, truncated form of HuAD10. Characterization of recombinant HuAD10 (rHuAD10) suggests that this **enzyme** has many of the properties (i.e. substrate specificity, metalloprotease activity, cellular location) expected for a physiologically relevant TNF-processing **enzyme**.

L4 ANSWER 25 OF 42 MEDLINE DUPLICATE 18
ACCESSION NUMBER: 97368283 MEDLINE
DOCUMENT NUMBER: 97368283 PubMed ID: 9223278
TITLE: Direct involvement of the ubiquitin-conjugating enzyme Ubc9/Hus5 in the degradation of IkappaBalpha.
AUTHOR: Tashiro K; Pando M P; Kanegae Y; Wamsley P M; Inoue S; Verma I M
CORPORATE SOURCE: Laboratory of Genetics, The Salk Institute for Biological Studies, La Jolla, CA 92037, USA.
SOURCE: PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (1997 Jul 22) 94 (15) 7862-7.
Journal code: 7505876. ISSN: 0027-8424.
PUB. COUNTRY: United States
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)

LANGUAGE: English
FILE SEGMENT: Priority Journals
OTHER SOURCE: GENBANK-U82627
ENTRY MONTH: 199708
ENTRY DATE: Entered STN: 19970908
Last Updated on STN: 20030222
Entered Medline: 19970827

AB . . . the cytoplasm in association with IkappaBalpha. In response to external signals, IkappaBalpha is phosphorylated, multi-ubiquitinated, and degraded by proteasomes, thereby **releasing** NF-kappaB/Rel proteins to migrate to the nucleus. We have cloned a mouse ubiquitin-conjugating **enzyme** (mE2), which associates with IkappaBalpha. mE2 is homologous to the yeast Ubc9/Hus5 ubiquitin-conjugating **enzyme**. A transdominant-negative mutant of mE2 had no effect on phosphorylation of IkappaBalpha, but delayed its degradation. Correspondingly, **tumor necrosis factor**-alpha-inducible NF-kappaB activity was diminished. We propose that mE2 is directly involved in the ubiquitin conjugation of IkappaBalpha, a pivotal step. . .

L4 ANSWER 26 OF 42 EMBASE COPYRIGHT 2003 ELSEVIER SCI. B.V.
ACCESSION NUMBER: 1998000496 EMBASE
TITLE: Effect of chloroqine, nitroquine, pyrimethamine on the release of TNF from macrophages.
AUTHOR: Meng D.-S.; Deng S.-F.; Lu J.-S.; Chen X.-H.; Hu Y.-M.
CORPORATE SOURCE: D.-S. Meng, Pharmacology Department, Third Military Medical University, Chongqing 630038, China
SOURCE: Chinese Pharmacological Bulletin, (1997) 13/5 (458-460). Refs: 7
ISSN: 1001-1978 CODEN: ZYTOE8
COUNTRY: China
DOCUMENT TYPE: Journal; Article
FILE SEGMENT: 030 Pharmacology
037 Drug Literature Index
LANGUAGE: Chinese
SUMMARY LANGUAGE: Chinese; English

AB AIM: To examine the effect of three anti-malaria drugs on the release of **tumor necrosis factor** (TNF) from rats peritoneal macrophages. METHOD **Enzyme**- linked immunosorbant assay. RESULTS: Erythrocyte infected by Plasmodium yoelii can induce TNF release from macrophages;). At 12th hour, the level of TNF reaches its peak. Chloroquine and nitro- quine have obvious inhibitive effect on the **releasing** of TNF, but pyrimethamme shows no effect. CONCLUSIONS: The results suggest that the inhibition of TNF may, be a part. . .

L4 ANSWER 27 OF 42 MEDLINE DUPLICATE 19
ACCESSION NUMBER: 97047721 MEDLINE
DOCUMENT NUMBER: 97047721 PubMed ID: 8926110
TITLE: Supernatants from Staphylococcus epidermidis grown in the presence of different antibiotics induce differential release of tumor necrosis factor alpha from human monocytes.
AUTHOR: Mattsson E; Van Dijk H; Verhoef J; Norrby R; Rollof J
CORPORATE SOURCE: Eijkman-Winkler Institute for Medical Microbiology, Infectious Diseases, and Inflammation, Utrecht, The Netherlands.
SOURCE: INFECTION AND IMMUNITY, (1996 Oct) 64 (10) 4351-5.
Journal code: 0246127. ISSN: 0019-9567.
PUB. COUNTRY: United States
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199611
ENTRY DATE: Entered STN: 19961219

Last Updated on STN: 19961219
Entered Medline: 19961114

AB Bacterial products from gram-positive bacteria, such as peptidoglycan, teichoic acid, and toxins, activate mononuclear cells to produce **tumor necrosis factor** alpha (TNF). The present study evaluated the release of soluble cell wall components from Staphylococcus epidermidis capable of inducing TNF. . . . or presence of human serum. After 18 h of incubation, monocyte supernatants were tested for the presence of TNF by **enzyme**-linked immunosorbent assay (ELISA). Supernatants from bacteria incubated with beta-lactam antibiotics induced higher TNF levels than those obtained from bacteria incubated. . . . by affinity depletion with vancomycin-Sepharose gel, were proportional to TNF release. Differences in the ability of individual antibiotics to generate TNF-**releasing** products from S. epidermidis were observed, the most potent antibiotics being penicillin and oxacillin.

L4 ANSWER 28 OF 42 MEDLINE DUPLICATE 20
ACCESSION NUMBER: 96245205 MEDLINE
DOCUMENT NUMBER: 96245205 PubMed ID: 8641833
TITLE: Expression and release of tumor necrosis factor-alpha by explants of mouse cornea.
AUTHOR: Sekine-Okano M; Lucas R; Rungger D; De Kesel T; Grau G E; Leuenberger P M; Rungger-Brandle E
CORPORATE SOURCE: Department of Ophthalmology, University Hospital, Geneva, Switzerland.
SOURCE: INVESTIGATIVE OPHTHALMOLOGY AND VISUAL SCIENCE, (1996 Jun) 37 (7) 1302-10.
Journal code: 7703701. ISSN: 0146-0404.
PUB. COUNTRY: United States
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199607
ENTRY DATE: Entered STN: 19960726
Last Updated on STN: 19960726
Entered Medline: 19960716

AB . . . agents widely used in the treatment of corneal disorders, the authors determined whether corneal cells are capable of expressing and **releasing tumor necrosis factor** -alpha (TNF alpha) on lipopolysaccharide (LPS) stimulation, and they investigated whether TNF alpha production can be modulated by pharmacologic agents. METHODS.. . . alpha after a 24-hour stimulation with LPS (1 microgram/ml) into the culture medium was determined both by bioassay and by **enzyme**-linked immunosorbent assay. Expression of TNF alpha mRNA after 6-hour stimulation was examined by polymerase chain reaction. Immunofluorescent staining on cryostat. . .

L4 ANSWER 29 OF 42 BIOSIS COPYRIGHT 2003 BIOLOGICAL ABSTRACTS INC.DUPLICATE 21
ACCESSION NUMBER: 1996:360023 BIOSIS
DOCUMENT NUMBER: PREV199699082379
TITLE: Identification of the proteolytic enzyme with cleaves human p75 TNF receptor in vitro.
AUTHOR(S): Katsura, Koichi; Park, Minha; Gatanaga, Maki; Yu, Ellen C.; Takishima, Kunio; Granger, Gale A.; Gatanaga, Tetsuya (1)
CORPORATE SOURCE: (1) Dep. Molecular Biol. Biochemistry, Univ. California, Irvine, Irvine, CA 92717-3900 USA
SOURCE: Biochemical and Biophysical Research Communications, (1996) Vol. 222, No. 2, pp. 298-302.
ISSN: 0006-291X.
DOCUMENT TYPE: Article
LANGUAGE: English
IT Miscellaneous Descriptors
ENZYME KINETICS; METALLOPROTEINASE-LIKE ENZYME; THP-1 MONOCYTE CELL;

TUMOR NECROSIS FACTOR RECEPTOR
RELEASING ENZYME

L4 ANSWER 30 OF 42 MEDLINE DUPLICATE 22
ACCESSION NUMBER: 95009733 MEDLINE
DOCUMENT NUMBER: 95009733 PubMed ID: 7925134
TITLE: Nerve growth factor in the anterior pituitary: regulation of secretion.
AUTHOR: Patterson J C; Childs G V
CORPORATE SOURCE: Department of Anatomy and Neuroscience, University of Texas Medical Branch, Galveston 77555-1043.
CONTRACT NUMBER: R01 DK-39553 (NIDDK)
SOURCE: ENDOCRINOLOGY, (1994 Oct) 135 (4) 1697-704.
Journal code: 0375040. ISSN: 0013-7227.
PUB. COUNTRY: United States
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Abridged Index Medicus Journals; Priority Journals
ENTRY MONTH: 199410
ENTRY DATE: Entered STN: 19941222
Last Updated on STN: 19970203
Entered Medline: 19941024

AB beta-Nerve growth **factor** (NGF) is a 26-kilodalton protein that may have a broader distribution and set of functions than its name implies. Its. . . study was to characterize further the NGF activity in AP cells, learn if it can be secreted, and determine the **factors** that may control secretion. NGF bioactivity was detected with assays of neurite outgrowth in PC12 **tumor** cells, and immunoreactivity was detected by an **enzyme**-linked immunoassay. AP cells secreted both bioactive and immunoreactive NGF at basal levels in vitro. In the **enzyme**-linked immunoassay, the anti-NGF recognized 2.5S NGF at a concentration of 0.10 pM, but it did not recognize brain-derived neurotrophic **factor**, neurotrophin-3 (NT-3), or NT-4, at concentrations as high as 10 nM. AP cells cultured for 6 days at 10(5) cells/200. . . beta) at a concentration of 1 nM caused up to a 2.5 fold increase in NGF secretion. In addition, GH **releasing** hormone, **tumor necrosis factor**-alpha, basic fibroblast growth **factor**, and forskolin all caused an inhibition of NGF secretion below basal levels. The evidence demonstrates the presence and secretion of. . . from AP cells. The fact that secretion is enhanced by IL-1 beta suggests that AP NGF may be a regulatory **factor** in the neuroendocrine-immune circuit.

L4 ANSWER 31 OF 42 MEDLINE DUPLICATE 23
ACCESSION NUMBER: 94102239 MEDLINE
DOCUMENT NUMBER: 94102239 PubMed ID: 8275970
TITLE: Physiological relevance of tumor necrosis factor in mediating macrophage-Leydig cell interactions.
AUTHOR: Moore C; Hutson J C
CORPORATE SOURCE: Department of Cell Biology and Anatomy, Texas Tech University Health Sciences Center, Lubbock 79430.
CONTRACT NUMBER: HD-26733 (NICHD)
SOURCE: ENDOCRINOLOGY, (1994 Jan) 134 (1) 63-9.
Journal code: 0375040. ISSN: 0013-7227.
PUB. COUNTRY: United States
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Abridged Index Medicus Journals; Priority Journals
ENTRY MONTH: 199402
ENTRY DATE: Entered STN: 19940218
Last Updated on STN: 19940218
Entered Medline: 19940207

AB Previously, we reported that testicular macrophages constitutively release **tumor necrosis factor** (TNF) in vitro and are

unresponsive to bacterial endotoxins [lipopolysaccharides (LPS)]. These properties are not typical of other tissue macrophages.. . . testicular interstitial fluid for TNF. Using the L929 cytotoxicity assay for TNF, we found that interstitial fluid contained a cytotoxic **factor**(s), but this bioactivity was not due to either authentic TNF or a TNF-like molecule acting through the TNF receptor. This. . . was cytotoxic to TNF-resistant L929 cells; and 3) there was no detectable TNF immunoreactivity in interstitial fluid, as measured by **enzyme** -linked immunosorbent assay. Therefore, we evaluated whether the release of TNF in vitro was induced by the isolation procedure, particularly by. . . macrophages obtained without the use of collagenase (agitation of testes in buffer) did not release TNF, but responded to the TNF-**releasing** effect of LPS. Exposure of peritoneal macrophages to collagenase resulted in constitutive TNF release in vitro and lack of responsiveness to LPS. There was no evidence that a non-TNF cytotoxic **factor** was released in the conditioned medium by any macrophage preparation. Taken together, our findings show that testicular macrophages do not. . .

L4 ANSWER 32 OF 42 MEDLINE DUPLICATE 24
ACCESSION NUMBER: 95116480 MEDLINE
DOCUMENT NUMBER: 95116480 PubMed ID: 7816774
TITLE: The present knowledge of the inflammatory process and the inflammatory mediators.
AUTHOR: Leirisalo-Repo M
CORPORATE SOURCE: Second Department of Medicine, University of Helsinki, Finland.
SOURCE: PHARMACOLOGY AND TOXICOLOGY, (1994) 75 Suppl 2 1-3. Ref: 12
Journal code: 8702180. ISSN: 0901-9928.
PUB. COUNTRY: Denmark
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
General Review; (REVIEW)
(REVIEW, TUTORIAL)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199502
ENTRY DATE: Entered STN: 19950217
Last Updated on STN: 19970203
Entered Medline: 19950206
AB . . . induction of P-selectin and PAF by histamine, thrombin and LTC4 contribute to the acute rolling of PMNs on endothelial surface. **Tumor necrosis factor** (TNF), interleukin-1 (IL-1) and lipopolysaccharide activate endothelial cells to synthesize interleukin-8 (IL-8), a potent chemotactic and proadhesive mediator for PMNs,. . . long-term adhesion between PMN and endothelium. After adhesion and migration to the focus of inflammation, PMNs induce inflammation by aggregating, **releasing** hydrolyzing **enzymes**, generating lipid peroxidation products such as prostaglandins and LTB4, and oxygen derived free radicals. In studies on the pathogenesis of. . .

L4 ANSWER 33 OF 42 MEDLINE DUPLICATE 25
ACCESSION NUMBER: 93233253 MEDLINE
DOCUMENT NUMBER: 93233253 PubMed ID: 7682629
TITLE: Human immunodeficiency virus type 1-specific cytotoxic T lymphocytes release gamma interferon, tumor necrosis factor alpha (TNF-alpha), and TNF-beta when they encounter their target antigens.
AUTHOR: Jassoy C; Harrer T; Rosenthal T; Navia B A; Worth J; Johnson R P; Walker B D
CORPORATE SOURCE: Infectious Disease Unit, Massachusetts General Hospital, Boston.
CONTRACT NUMBER: AI 26463 (NIAID)

AI 28568 (NIAID)
AI 30914 (NIAID)

SOURCE: JOURNAL OF VIROLOGY, (1993 May) 67 (5) 2844-52.
Journal code: 0113724. ISSN: 0022-538X.
PUB. COUNTRY: United States
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals; AIDS
ENTRY MONTH: 199305
ENTRY DATE: Entered STN: 19930604
Last Updated on STN: 19970203
Entered Medline: 19930517

AB . . . study, we evaluated the ability of HIV-1-specific cytotoxic T-lymphocyte (CTL) clones derived from seropositive persons to release gamma interferon (IFN-gamma), **tumor necrosis factor** alpha (TNF-alpha), and TNF-beta upon contact with target cells presenting viral antigen. Peripheral blood- and cerebrospinal fluid-derived HIV-1-specific CD3+ CD4-. . . cell lines sensitized with synthetic HIV-1 peptides containing the epitopes recognized by these CTL. Cytokine production was measured by specific **enzyme**-linked immunosorbent assay of culture supernatant fluid. HIV-1-specific CTL clones directed at envelope, Gag, reverse transcriptase, and Nef epitopes specifically released. . . these cells. These studies indicate that in addition to mediating direct cytotoxicity, HIV-1-specific CTL may affect other immune responses by **releasing** IFN-gamma, TNF-alpha, and TNF-beta. Elevated levels of these cytokines which have been detected in serum and cerebrospinal fluid of infected. . .

L4 ANSWER 34 OF 42 MEDLINE
ACCESSION NUMBER: 92314358 MEDLINE
DOCUMENT NUMBER: 92314358 PubMed ID: 1319763
TITLE: Hormonal regulation of inflammatory cell cytokine transcript and bioactivity production in response to endotoxin.
AUTHOR: Doherty G M; Jensen J C; Buresh C M; Norton J A
CORPORATE SOURCE: Surgical Metabolism Section, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892.
SOURCE: CYTOKINE, (1992 Jan) 4 (1) 55-62.
Journal code: 9005353. ISSN: 1043-4666.
PUB. COUNTRY: United States
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199208
ENTRY DATE: Entered STN: 19920815
Last Updated on STN: 19920815
Entered Medline: 19920804

AB **Tumor necrosis factor** (TNF), interleukin 1 (IL-1) and interleukin 6 (IL-6) are central mediators of the inflammatory response. We investigated the modulation of. . . to various concentrations of hormones followed by lipopolysaccharide (LPS, 10 micrograms/ml). TNF, IL-1 and IL-6 production were assessed by bioassays, **enzyme**-linked immunosorbent assays (ELISA) or Western blot, and specific RNA transcripts by Northern blot. Hydrocortisone in concentrations as low as 10. . . a post-transcriptional level. ACTH and insulin increased supernatant levels of IL-6 produced in response to LPS without altering available transcripts. Corticotrophin-**releasing factor** (CRF), epinephrine and glucagon had no effect on supernatant levels of cytokine. Thus, physiological and pharmacological concentrations of hydrocortisone had. . .

L4 ANSWER 35 OF 42 MEDLINE DUPLICATE 26
ACCESSION NUMBER: 91149381 MEDLINE
DOCUMENT NUMBER: 91149381 PubMed ID: 1671798

TITLE: Growth factor-mediated regulation of aromatase activity in human skin fibroblasts.
AUTHOR: Emoto N; Ling N; Baird A
CORPORATE SOURCE: Department of Molecular and Cellular Growth Biology, Whittier Institute for Diabetes and Endocrinology, La Jolla, California 92037.
CONTRACT NUMBER: DK-18811 (NIDDK)
NS-28121 (NINDS)
SOURCE: PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE, (1991 Mar) 196 (3) 351-8.
Journal code: 7505892. ISSN: 0037-9727.
PUB. COUNTRY: United States
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199104
ENTRY DATE: Entered STN: 19910419
Last Updated on STN: 20000303
Entered Medline: 19910402

AB We investigated the effects of various hormones and growth **factors** on aromatase activity in cultured human skin fibroblasts. Several potential trophic **factors** were tested for their ability to modify basal aromatase activity or the response to dibutyryladenosine 3',5'-cyclic monophosphate and dexamethasone because. . . identified that is responsible for stimulating aromatase activity in the periphery, and (ii) dexamethasone and cAMP analogs can increase this **enzyme** 's activity in fibroblasts. The effect of insulin and insulin-like growth **factors** were examined in closer detail because of the clinical association between insulin and hyperandrogenism. Pituitary hormones and hypothalamic **releasing factors**, such as human ACTH (10 nM), beta-endorphin (10 nM), beta-lipotropin (10 nM), alpha-MSH (10 nM), gamma 3-MSH (10 nM), ovine. . . ovine follicle-stimulating hormone (10 ng/ml), ovine thyroid-stimulating hormone (10 ng/ml), rat growth hormone (10 ng/ml), rat prolactin (10 ng/ml), rat corticotropin-**releasing factor** (10 nM), luteinizing hormone-**releasing factor** (10 nM), thyrotropin-**releasing factor** (10 nM), human growth hormone-**releasing factor** (10 nM), and somatostatin (10 nM), have no significant effects on aromatase activity. Porcine inhibin A (10 ng/ml) and porcine activin AB (10 ng/ml), two ovarian hormones with structural transforming homology to transforming growth **factor**-beta, also have no effect on aromatase activity. Although basic fibroblast growth **factor** (1-100 ng/ml), acidic fibroblast growth **factor** (1 ng/ml), epidermal growth **factor** (1 ng/ml), platelet-derived growth **factor** (1 ng/ml), **tumor necrosis factor** (1 ng/ml), and transforming growth **factor**-beta 1 (1 ng/ml) have no effect on basal aromatase activity in human skin fibroblasts, all of these growth **factors** inhibited the ability of dibutyryladenosine 3',5'-cyclic monophosphate to stimulate aromatase activity. In contrast, both insulin (100 pg/ml-10 ng/ml) and insulin-like growth **factor**-1 (1-100 ng/ml) had no effect on cAMP-stimulated aromatase but potentiated the action of dexamethasone (100 nM). Thus, there is a. . . to speculate that the hyperandrogenism that is often associated with insulin resistance may be due to a combination of growth **factor**-mediated inhibition of aromatase activity and the failure of peripheral tissues to respond to insulin and metabolize androgens to estrogens.

L4 ANSWER 36 OF 42 MEDLINE DUPLICATE 27
ACCESSION NUMBER: 92044032 MEDLINE
DOCUMENT NUMBER: 92044032 PubMed ID: 1940574
TITLE: An inhibitor of tumor necrosis factor found in pleural effusions.
AUTHOR: Baughman R P; Lower E E
CORPORATE SOURCE: Department of Medicine, University of Cincinnati Medical

Center, Ohio.
SOURCE: JOURNAL OF LABORATORY AND CLINICAL MEDICINE, (1991 Oct) 118 (4) 326-31.
Journal code: 0375375. ISSN: 0022-2143.
PUB. COUNTRY: United States
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Abridged Index Medicus Journals; Priority Journals
ENTRY MONTH: 199112
ENTRY DATE: Entered STN: 19920124
Last Updated on STN: 19920124
Entered Medline: 19911210

AB **Tumor necrosis factor** (TNF) has significant biologic effects. Inhibitors of TNF have been isolated from urine and blood. We studied pleural fluid from 22 patients with benign or malignant effusions. Pleural macrophages from these effusions were capable of **releasing** TNF, especially when stimulated with lipopolysaccharide. The cell-free supernatant from some of these pleural effusions contained an inhibitor of TNF.. . . and unaffected by dialysis, and the molecular weight of at least one of the inhibitors was 60 to 80,000 daltons. **Enzyme** digestion studies were consistent with a protein portion being the major determinant of activity. We conclude that some malignant effusions. . .

L4 ANSWER 37 OF 42 MEDLINE DUPLICATE 28
ACCESSION NUMBER: 91297488 MEDLINE
DOCUMENT NUMBER: 91297488 PubMed ID: 2068574
TITLE: Prolactin, immunoregulation, and autoimmune diseases.
AUTHOR: Jara L J; Lavalle C; Fraga A; Gomez-Sanchez C; Silveira L H; Martinez-Osuna P; Germain B F; Espinoza L R
CORPORATE SOURCE: Department of Internal Medicine, University of South Florida College of Medicine, Tampa.
SOURCE: SEMINARS IN ARTHRITIS AND RHEUMATISM, (1991 Apr) 20 (5) 273-84. Ref: 93
Journal code: 1306053. ISSN: 0049-0172.
PUB. COUNTRY: United States
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
General Review; (REVIEW)
(REVIEW, TUTORIAL)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199108
ENTRY DATE: Entered STN: 19910901
Last Updated on STN: 19910901
Entered Medline: 19910814

AB . . . of the immune system synthesize prolactin and express mRNA and receptors for that hormone. Interleukin 1, interleukin 6, gamma interferon, **tumor necrosis factor**, platelet activator **factor**, and substance P participate in the release of prolactin. This hormone is involved in the pathogenesis of adjuvant arthritis and. . . interleukin 2 receptors on the surface of lymphocytes. Prolactin stimulates ornithine decarboxylase and activates protein kinase C, which are pivotal **enzymes** in the differentiation, proliferation, and function of lymphocytes. Cyclosporine A interferes with prolactin binding to its receptors on lymphocytes. Hyperprolactinemia. . . found in patients with systemic lupus erythematosus. Fibromyalgia, rheumatoid arthritis, and low back pain patients present a hyperprolactinemic response to thyrotropin-**releasing** hormone. Experimental autoimmune uveitis, as well as patients with uveitis whether or not associated with spondyloarthropathies, and patients with psoriatic. . .

L4 ANSWER 38 OF 42 MEDLINE DUPLICATE 29
ACCESSION NUMBER: 92136480 MEDLINE

DOCUMENT NUMBER: 92136480 PubMed ID: 1663842
TITLE: Role of leukocyte-derived pro-opiomelanocortin peptides in endotoxic shock.
AUTHOR: Harbour D V; Galin F S; Hughes T K; Smith E M; Blalock J E
CORPORATE SOURCE: Department of Psychiatry and Behavioral Sciences, University of Texas Medical Branch, Galveston 77550.
CONTRACT NUMBER: 1-R29-NS-27546 (NINDS)
SOURCE: CIRCULATORY SHOCK, (1991 Nov) 35 (3) 181-91. Ref: 68
Journal code: 0414112. ISSN: 0092-6213.
PUB. COUNTRY: United States
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
General Review; (REVIEW)
(REVIEW, TUTORIAL)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199203
ENTRY DATE: Entered STN: 19920329
Last Updated on STN: 19920329
Entered Medline: 19920310

AB . . . including immunological stimuli such as bacterial lipopolysaccharide (LPS; endotoxin), virus infection (Newcastle virus; NDV), and the more classical neuroendocrine stimuli corticotropin-**releasing** hormone (CRH). We have proposed that the production of END by the peripheral immune system contributes to the pool of. . . induces a novel protease that functions optimally at pH 5 to cleave ACTH 1-39 into ACTH 1-22 to 1-26. This **enzyme** is present in LPS, but not mock or CRH-induced B cells from LPS-sensitive mice. The LPS-resistant mice did not possess this **enzyme** and therefore produced only the high-molecular-weight pro-opiomelanocortin (POMC)-like molecule. The inability to produce ACTH and END, presumably by their inability. . . also may play an indirect role in orchestrating the pathophysiologic response, since both ACTH and END were shown to induce **tumor necrosis factor** (TNF). Our data strongly suggest that lymphocyte POMC peptides ACTH and END are important mediators in the overall response to. . .

L4 ANSWER 39 OF 42 MEDLINE DUPLICATE 30
ACCESSION NUMBER: 90297254 MEDLINE
DOCUMENT NUMBER: 90297254 PubMed ID: 2360646
TITLE: Liberation of a neutrophil enzyme-releasing peptide from the surface of human alveolar macrophages.
AUTHOR: Miller E J; MacArthur C K; Gray L D; Cohen A B
CORPORATE SOURCE: Department of Biochemistry, University of Texas Health Center, Tyler 75710.
CONTRACT NUMBER: R01-HL43650 (NHLBI)
SOURCE: AMERICAN JOURNAL OF PHYSIOLOGY, (1990 Jun) 258 (6 Pt 1) L328-33.
Journal code: 0370511. ISSN: 0002-9513.
PUB. COUNTRY: United States
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199008
ENTRY DATE: Entered STN: 19900907
Last Updated on STN: 19900907
Entered Medline: 19900802

AB The human alveolar macrophage product, **enzyme-releasing** peptide (ERP), has a molecular mass of 8,000 Da, and releases azurophilic and specific granule constituents from neutrophils. A murine monoclonal anti-ERP antibody (12E10H), previously used to show a lack of antigenic identity between ERP and C5a, interleukin 1, **tumor necrosis factor**, and gamma-interferon, showed no cross-reactivity with interleukin 8. 12E10H and a fluorescein-labeled second antibody were used to visualize ERP on. . . readhere to plastic

and exclude trypan blue. Dilution of the trypsin-derived ERP released myeloperoxidase from cytochalasin-B-treated neutrophils dose dependently. The **enzyme-releasing** ability of the trypsin-derived material was removed by immunoprecipitation using antibody 12E10H bound to Staphylococcal protein A Sepharose 4B. The. . .

L4 ANSWER 40 OF 42 MEDLINE DUPLICATE 31
ACCESSION NUMBER: 88060436 MEDLINE
DOCUMENT NUMBER: 88060436 PubMed ID: 3680945
TITLE: A peptide secreted by human alveolar macrophages releases neutrophil granule contents.
COMMENT: Erratum in: J Immunol 1988 Mar 1;140(5):1713
AUTHOR: MacArthur C K; Miller E J; Cohen A B
CORPORATE SOURCE: Department of Biochemistry, University of Texas Health Center at Tyler 75710.
CONTRACT NUMBER: RO1-HL34745 (NHLBI)
SOURCE: JOURNAL OF IMMUNOLOGY, (1987 Nov 15) 139 (10) 3456-62. Journal code: 2985117R. ISSN: 0022-1767.
PUB. COUNTRY: United States
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Abridged Index Medicus Journals; Priority Journals
ENTRY MONTH: 198712
ENTRY DATE: Entered STN: 19900305
Last Updated on STN: 19970203
Entered Medline: 19871222

AB A monoclonal antibody was developed against an 8,000-kDa **enzyme-releasing** peptide (ERP) released from human alveolar macrophages. ERP was isolated on an immunoaffinity column containing the antibody bound to staphylococcal. . . is not changed by plastic adherence, phagocytosis, calcium ionophore, or phorbol esters. The peptide was not antigenically similar to interferon-gamma, **tumor necrosis factor**, or interleukin 1 alpha or 1 beta. The release of constituents from azurophilic and specific granules was the main identified. . . is a secretagogue for human neutrophils under conditions which may be encountered in the lungs during certain disease states. Proteolytic **enzymes** which are free in the lungs may release the peptide and lead to the secretion of neutrophil **enzymes**.

L4 ANSWER 41 OF 42 MEDLINE DUPLICATE 32
ACCESSION NUMBER: 88057583 MEDLINE
DOCUMENT NUMBER: 88057583 PubMed ID: 3679539
TITLE: Bacterial-lipopolysaccharide-induced release of lactoferrin from human polymorphonuclear leukocytes: role of monocyte-derived tumor necrosis factor alpha.
AUTHOR: Koivuranta-Vaara P; Banda D; Goldstein I M
CORPORATE SOURCE: Rosalind Russell Arthritis Research Laboratory, Department of Medicine, University of California, San Francisco 94143-0868.
CONTRACT NUMBER: AR-36949 (NIAMS)
HL-19155 (NHLBI)
SOURCE: INFECTION AND IMMUNITY, (1987 Dec) 55 (12) 2956-61. Journal code: 0246127. ISSN: 0019-9567.
PUB. COUNTRY: United States
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 198801
ENTRY DATE: Entered STN: 19900305
Last Updated on STN: 19970203
Entered Medline: 19880106

AB . . . as with the duration of incubation (2 to 60 min) and was not accompanied by significant release of the cytoplasmic **enzyme**

lactate dehydrogenase. LPS-induced release of lactoferrin from PMN was augmented significantly when cell suspensions were supplemented with additional monocytes and lymphocytes. Only monocytes, however, secreted significant amounts of lactoferrin-**releasing** activity (in a time- and concentration-dependent manner) when incubated separately with LPS. Lactoferrin-**releasing** activity was heat (80 degrees C for 15 min) labile, eluted after chromatography on Sephadex G-100 with an apparent molecular weight of approximately 60,000, and was inhibited by antibodies to **tumor necrosis factor** alpha.
Thus, LPS-induced noncytotoxic release of lactoferrin from human PMN suspended in serum-free buffer is mediated, at least in part, by **tumor necrosis factor** alpha derived from contaminating monocytes.

L4 ANSWER 42 OF 42 CAPLUS COPYRIGHT 2003 ACS
ACCESSION NUMBER: 1987:79381 CAPLUS
DOCUMENT NUMBER: 106:79381
TITLE: Low-temperature pulverization of hormones and other substances
INVENTOR(S): Fujioka, Takaharu; Sato, Shigeji; Takada, Yoshihiro
PATENT ASSIGNEE(S): Sumitomo Pharmaceuticals Co., Ltd., Japan
SOURCE: Jpn. Kokai Tokkyo Koho, 3 pp.
CODEN: JKXXAF
DOCUMENT TYPE: Patent
LANGUAGE: Japanese
FAMILY ACC. NUM. COUNT: 1
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
JP 61236721	A2	19861022	JP 1985-77249	19850411

PRIORITY APPLN. INFO.: JP 1985-77249 19850411

AB Heat-labile prostaglandins, prostacyclins, growth hormone, growth hormone-**releasing factor**, somatomedin, plasminogen activator, interferons, interleukins, **tumor necrosis factor**, **enzymes**, etc. are subjected to low-temp. pulverization to avoid inactivation. Thus, 10 mg human growth hormone-releasing factor was dissolved in 800 mL 25% human serum albumin soln., freeze dried, cooled with dry ice, and pulverized at -20.degree..

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Fr m: Murphy, Joseph
Sent: Monday, March 03, 2003 10:49 AM
T : STIC-Biotech/ChemLib
Subject: 09712813

Please send me the following references:

Hwang C, Gatanaga M, Granger GA, Gatanaga T. Mechanism of release of soluble forms of tumor necrosis factor/lymphotoxin receptors by phorbol myristate acetate-stimulated human THP-1 cells in vitro. J Immunol. 1993 Nov 15;151(10):5631-8.

Vey E, Burger D, Dayer JM. Expression and cleavage of tumor necrosis factor-alpha and tumor necrosis factor receptors by human monocytic cell lines upon direct contact with stimulated T cells. Eur J Immunol. 1996 Oct;26(10):2404-9.

Bjornberg F, Lantz M, Olsson I, Gullberg U. Mechanisms involved in the processing of the p55 and the p75 tumor necrosis factor (TNF) receptors to soluble receptor forms.
Lymphokine Cytokine Res. 1994 Jun;13(3):203-11

Bjornberg F, Lantz M, Gullberg U. Metalloproteases and serineproteases are involved in the cleavage of the two tumour necrosis factor (TNF) receptors to soluble forms in the myeloid cell lines U-937 and THP-1. Scand J Immunol. 1995 Oct;42(4):418-24.

Gabay C. Cytokine inhibitors in the treatment of rheumatoid arthritis.
Expert Opin Biol Ther. 2002 Feb;2(2):135-49.

Taylor PC. Anti-TNF therapy for rheumatoid arthritis and other inflammatory diseases.
Mol Biotechnol. 2001 Oct;19(2):153-68.

LaDuca JR, Gaspari AA. Targeting tumor necrosis factor alpha. New drugs used to modulate inflammatory diseases. Dermatol Clin. 2001 Oct;19(4):617-35

Thanks a lot...

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Mechanism of Release of Soluble Forms of Tumor Necrosis Factor/Lymphotoxin Receptors by Phorbol Myristate Acetate-Stimulated Human THP-1 Cells in vitro

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*Department of Molecular Biology and Biochemistry, University of California at Irvine, Irvine, CA 92717; and the
†Memorial Cancer Institute, Long Beach Memorial Hospital, Long Beach, CA 90801

ABSTRACT. The mechanism involved in the release of the soluble forms of 55 and 75 kDa TNF and lymphotoxin (LT) membrane receptors was studied in a continuous human monocytic cell line, THP-1, in vitro. THP-1 cells were found to spontaneously release soluble forms of both 55 and 75 kDa TNF/LT receptors. Release was up-regulated by PMA, and optimal release was achieved at 10^{-8} M PMA. Serine protease inhibitors such as PMSF,3,4 dichloroisocoumarin, Nα-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), and N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) were found to inhibit the production of both soluble TNF/LT receptors. PMSF (2 mM) also blocked receptors shedding from paraformaldehyde-fixed THP-1 cells coincubated with conditioned media from PMA-stimulated THP-1 cells. Colchicine at 1 and 10 μM stimulated the production of both soluble TNF/LT receptors, but the PMA-induced release of both soluble TNF/LT receptors was inhibited. It appears that the PMA-induced release of soluble TNF/LT receptors involves serine proteases in the extracellular space where the soluble parts of the TNF/LT receptors are cleaved directly off the cell membrane. *Journal of Immunology*, 1993, 151: 5631.

TNF-α and LT[2] are related cytokines produced by activated macrophages and lymphocytes (1–3). Two distinct TNF/LT membrane receptors of 55 and 75 kDa have been isolated, sequenced, and cloned (4–6). Both receptors are anchored on the cell membrane by a single transmembrane region. The extracellular domains share 28% homology, whereas the intracellular domains share none. The Kd values of 55 and 75 KDa receptors for TNF are approximately 0.5 and 0.1 nM, respectively (4–6). The specific function of each receptor is still under investigation. Soluble forms of both receptors of 30 to 40 kDa have been identified in urine from normal individuals and patients with chronic inflammatory diseases (7, 8) and in serum from patients with various types of cancer (9, 10). These soluble forms are the NH_2-terminal extracellular domains of these membrane receptors and have the ability to bind to and inactivate human TNF and LT in vitro and in vivo.

The role of soluble TNF/LT receptors in immunologic reactions is not clear. It has been suggested that they may have important roles in the regulation of TNF and LT activity in vivo. They may help cancer cells to evade immunosurveillance by blocking TNF and LT activity (11). However, soluble receptors were also found to stabilize TNF activity in vitro at low TNF:receptor ratios (12). Thus, these molecules may have both positive and negative regulatory effects. The mechanism by which soluble TNF/LT receptors are released from cells is still largely unknown. Immunoprecipitation studies by Lantz et al. (13) suggested that the soluble form receptor derived from the 55 kDa TNF/LT receptor is generated by proteolytic cleavage of the membrane receptor. Porteu and Nathan (14) showed FMLP can stimulate human neutrophils to release soluble TNF/LT receptors in vitro. Porteu et al. (15) also found that elastase in the azurophil granules of neutrophils could

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[1] Address correspondence and reprint requests to Dr. Tetsuya Gatanaga, Department of Molecular Biology and Biochemistry, University of California at Irvine, Irvine, CA 92717.

[2] Abbreviations used in this paper: LT, lymphotoxin; TLCK, Nα-*p*-tosyl-L-lysine chloromethyl ketone; TPCK, N-tosyl-L-phenylalanine chloromethyl ketone.

cleave a 32-kDa fragment from the membrane-bound 75 kDa receptor. However, inhibitors of elastase could not block FMLP-induced receptor release. Thus, the role of elastase in the release of soluble TNF/LT receptors by these cells in vitro is not clear.

In this study, PMA-stimulated human THP-1 cells were used to study the mechanism of release of soluble TNF/LT receptors. The data suggest that the PMA-induced release is probably mediated by the serine proteases that cleave the NH_2-terminal extracellular portion of TNF/LT receptors off the cell membrane extracellularly.

Materials and Methods

Cell line and reagents

THP-1, a human monocytic cell line that grows in suspension, was purchased from American Type Culture Collection (Rockville, MD). These cells were passed twice a week in RPMI 1640 (GIBCO Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated FBS (Irvine Scientific, Santa Ana, CA). PMA and the various inhibitors colchicine, PMSF,3,4 dichloroisocoumarin, TLCK, and TPCK were purchased from Sigma Chemical Co. (St. Louis). Recombinant forms of the soluble 55 and 75 kDa human TNF/LT receptors were kindly provided by Synergen (Boulder, CO). Rat anti-human 75 kDa TNF receptor mAb was kindly provided by Immunex (Immunex Corp., Seattle, WA).

Cell stimulation and supernatant collection

THP-1 cells from 3 to 4 day cultures were pelleted by centrifugation at 400 × *g* for 10 min and resuspended to a density of 8×10^5 cells/ml in RPMI 1640 supplemented with 10% FBS in a 50 ml polypropylene centrifuge tube (Corning Glass Works, Corning, NY). One milliliter aliquots were dispensed in each well of a 24-well polystyrene plate (Corning Glass Works). One hundred times concentrated stock solutions of PMA were added to each well in a volume of 10 μl, and the plate was incubated at 37°C in a CO_2 incubator for 8 h. The supernatants were then collected for the dose-response study. In the time course study, PMA (10^{-8} M) was added to each well, and supernatants were collected at various time points after incubation at 37°C in a CO_2 incubator. In the studies with inhibitors, supernatant were collected after 6 h incubation of the cells with both PMA and the specific inhibitor. Supernatants were cleared of cells by centrifugation at 500 × *g* for 5 min and were assayed for the concentration of soluble TNF/LT receptors by ELISA, as described in the following section.

ELISA for soluble 55 and 75 kDa TNF receptors

Anti-TNF receptor sera was generated by immunization of rabbits with human recombinant 55 or 75 kDa receptors by the method of Yamamoto et al. (16). The IgG fraction of rabbit serum was purified using a protein G (Pharmacia Fine Chemicals, Uppsala, Sweden) affinity column by the method of Ey et al. (17). The IgG fraction was then labeled with horseradish peroxidase (Sigma Chemical Co.) as described (18, 19). The specificity of the antisera used in this study has been checked. No cross-reactivity was observed when antisera were tested against each TNF receptor, and no reactivity was observed when antisera were tested against human recombinant forms of LT, TNF, IFN-γ, IL-1β, IL-2, IL-4, and IL-6. To start the ELISA, 100 μl of unlabeled IgG (5 μg/ml in 0.05 M sodium bicarbonate buffer, pH 9.5) was added to each well of a 96-well ELISA plate (Corning Glass Works) and incubated at 4°C overnight. Individual wells were washed three times with 300 μl 0.2% Tween 20 in PBS. One hundred microliter samples and recombinant TNF/LT receptor standards were then added to each well. The plates were incubated at 37°C for 3 h. The wells were then washed and 100 μl of peroxidase-labeled IgG was added. The substrate (2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (Pierce, Rockford, IL); hydrogen peroxide, 30% (Fisher Scientific, Fair Lawn, NJ) was prepared as instructed by the manufacturer. After 1 h incubation at 37°C with peroxidase-labeled IgG, wells were washed and 100 μl of substrate solution was added to each well. The plates were incubated at room temperature for 20 min and the results were obtained by measuring OD405 on an EAR 400 AT plate reader (SLT-Lab Instruments, Salzburg, Austria). The concentration of soluble receptors in each sample was calculated from the regression line computed by known standards for each receptor. The background absorbance was approximately 0.17 for the blank. The concentration of standards ranged from 125 pg/ml to 10 ng/ml. The highest values of the standard curve were approximately 1.3. Most of the R^2 values of the linear regression were greater than 0.99.

Assessment of TNF receptor release from paraformaldehyde-fixed THP-1 cells

THP-1 cells (approximately 10^8 cells) from 3 to 4 day cultures were pelleted as described above in six 50 ml polypropylene centrifuge tubes. Forty milliliters of PBS was added to each tube to resuspend the cells. The cells in each tube were pelleted again and resuspended in 10 ml PBS. Ten milliliters of 2% paraformaldehyde (Polysciences, Warrington, PA) in PBS was then added to each tube and the resulting suspension was incubated for 20 min at room temperature. The cells were then pelleted and washed with 40 ml of PBS for three times. The pellets were then resuspended, pooled together in 10 ml PBS, and stored at 4°C until use.

THP-1 cells (8×10^5 cells/ml) were stimulated with 10^{-8}

M PMA in a T150 polystyrene tissue culture flask for 24 h at 37°C. The supernatant from this culture was collected and cleared of cells by centrifugation at 400 × *g* for 10 min. It was then preincubated with or without 2 mM PMSF for 1 h at 37°C. An aliquot of PMSF stock solution was then added to the supernatant to make the final PMSF concentration 4 mM. Five hundred microliters of supernatant were combined with 500 μl of the paraformaldehyde-fixed THP-1 cells in individual wells of a 24-well polystyrene plate. After incubating at 37°C for 3 to 4 h, the supernatants were collected and cleared of cells by centrifugation at 500 × *g* for 5 min. The concentration of soluble TNF/LT receptors was then obtained by ELISA.

Molecular mass of soluble TNF/LT receptors

THP-1 cells (8×10^5 cells/ml) in 500 ml RPMI 1640 were stimulated with 10^{-8} M PMA overnight. The supernatant was collected then cleared of cells and concentrated to 15 ml by Amicon membrane filtration system using a filter with m.w. cut at 10 kDa (Amicon, Beverly, MA). Three milliliters of the concentrated supernatant was loaded on a Sephadex G100 (Pharmacia Fine Chemicals) column (2.5 × 44 cm) equilibrated in PBS buffer. The column was eluted with PBS at flow rate of 0.5 ml/min and the eluate was collected in 3.7-ml fractions. All fractions were assayed by ELISA to determine the presence of soluble TNF/LT receptors. The peak fractions of the soluble TNF/LT receptors from six runs were pooled to obtain a final volume of approximately 240 ml each. The pooled eluates were concentrated in two steps to approximately 750 μl, first with the Amicon membrane filtration system, followed by Centriprep 10 concentrator (m.w. cut at 10 kDa) (Amicon). The soluble receptor from the 55 kDa TNF/LT receptor was further purified by running the concentrated eluate on a 10% preparative SDS-PAGE in Model 291 Prep Cell using the procedures provided by the manufacturer (Bio-Rad, Richmond, CA). Volumes of the concentrated samples containing 5 ng of soluble receptor from either the 55 or 75 kDa receptor were loaded and run on a 1.5 mm 10% acrylamide slab gel as described (20). The proteins were then electrophoretically transferred to a polyvinylidene difluoride membrane (Immobilon, Millipore, Bedford, MA). Immunostaining was performed using the biotin-streptavidine system (Amersham, Amersham, UK) and peroxidase substrate kit DAB (Vector Laboratories, Burlingame, CA).

Results

Release of soluble 55 and 75 kDa TNF/LT receptors by PMA-stimulated THP-1 cells in vitro

THP-1 cells were cultured alone or with different concentrations of PMA for 8 h in a 24-well plate in a CO_2 incubator. Cell-free supernatants were assayed for soluble forms of both 55 and 75 kDa TNF/LT receptors by ELISA. As shown in the control columns of Figures 1*A* and *B*, THP-1 cells spontaneously released soluble forms of both receptors. However, the level of soluble TNF/LT receptors increased with increasing concentrations of PMA. The optimal effect of PMA in inducing TNF/LT receptors release was reached at 10^{-8} M. Next, 10^{-8} M PMA was used to stimulate the THP-1 cells and the time course of release was observed (Fig. 1*C* and *D*).

M.W. of soluble TNF/LT receptors in supernatants from PMA-stimulated THP-1 cells

The soluble TNF/LT receptors were partially purified by gel filtration and preparative SDS-PAGE. Partially purified samples were then subjected to Western blotting. Each soluble TNF/LT receptor showed specific single band. The soluble TNF/LT receptor derived from the 55 kDa membrane receptor corresponds with a 30-kDa band (Fig. 2*A*, lane 1), whereas that from the 75 kDa membrane receptor corresponds with a 40-kDa band (Fig. 2*B*, lane 1). To demonstrate the specificity of these bands, immunostaining was performed in the presence of excess amount of free recombinant extracellular portion of each TNF/LT receptor. Both bands disappeared in the presence of competing molecules (Fig. 2*A* and *B*, lane 2).

A major concern of the specificity of the rabbit anti-human 75 kDa TNF receptor polyclonal antibody was brought up by a major nonspecific band observed in the 67 kDa region in Figure 2*B*. One possible problem is that this nonspecific protein is also secreted by macrophages and is up-regulated by PMA. To validate the ELISA data, a rat anti-human 75 kDa TNF receptor mAb was used to establish another ELISA. The difference between data obtained from the polyclonal and the monoclonal ELISA was approximately 5% (data not shown). The values obtained from the monoclonal ELISA were actually higher than those from the polyclonal ELISA. These findings eliminate the possibilities that the polyclonal ELISA was picking up changes of a nonspecific protein.

Effects of serine protease inhibitors on the release of soluble TNF/LT receptors by THP-1 cells in vitro

Several serine protease inhibitors were tested for their effects on the release of soluble TNF/LT receptors. In general, a dose-dependent inhibition of soluble TNF/LT receptors release could be demonstrated. As shown in Table 1, the PMA-induced release of soluble receptor of the 55 and 75 kDa TNF/LT receptors decreased as the dose of inhibitors increased. 3,4 Dichloroisocoumarin failed to inhibit the spontaneous release of both soluble TNF/LT receptors, but showed significant inhibition on the PMA-induced release (for 55 kDa, $p < 0.001$ for both concentrations; for 75 kDa, $p < 0.02$ at 0.01 mM and

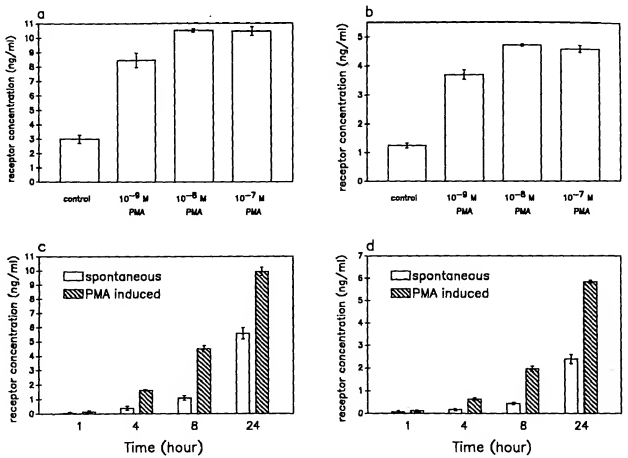

FIGURE 1. Dose response and time course of PMA-stimulated release of soluble TNF receptors. THP-1 cells were cultured with different concentrations of PMA for 8 h as described in *Materials and Methods*. The concentration of the soluble receptors was measured by ELISA (*A*, 55 kDa; *B*, 75 kDa). The time course was done with PMA at 10^{-8} M (*C*, 55 kDa; *D*, 75 kDa).

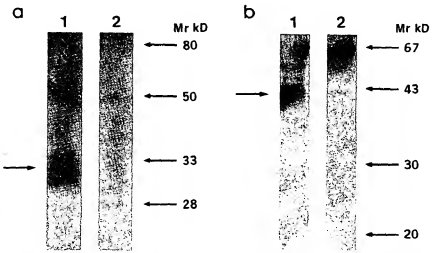

FIGURE 2. M.w. of the soluble TNF/LT receptors. Soluble TNF/LT receptors were partially purified from the supernatant collected from PMA-stimulated THP-1 cells by gel filtration and preparative SDS-PAGE, as described in *Materials and Methods*. The partially purified samples were subjected to Western blotting. *A*: soluble form receptor of 55 kDa TNF/LT receptor. *B*: soluble form receptor of 75 kDa TNF/LT receptor. Lane 1, samples alone: Lane 2, 100-fold excess of free recombinant soluble TNF/LT receptors coincubated with the anti-TNF/LT receptor antibodies.

$p < 0.01$ at 0.10 mM). TPCK at 0.1 mM generated a pattern similar to that of 3,4 dichloroisocoumarin. TPCK at 1 mM appeared to shut off the release of soluble TNF/LT receptors independent of PMA stimulation. However, the effect was secondary to the cytotoxicity of TPCK that killed all the cells at 1 mM. TLCK (0.01 and 0.1 mM) showed the

Table I
Effect of serine protease inhibitors on the release of soluble TNF receptors

	Spontaneous (ng/ml)		PMA-Induced (ng/ml)	
	55 kDa	75 kDa	55 kDa	75 kDa
Control	1.26 ± 0.23	1.22 ± 0.20	5.56 ± 0.32	4.60 ± 0.45
PMSF (mM)				
0.25	1.03 ± 0.04	1.10 ± 0.09	4.64 ± 0.74	4.36 ± 0.61
1.00	0.94 ± 0.12	1.04 ± 0.12	3.88 ± 0.36	3.64 ± 0.44
4.00	0.51 ± 0.24	0.65 ± 0.21	0.78 ± 0.69	0.87 ± 0.56
3,4 Dichloroisocoumarin (mM)				
0.01	1.20 ± 0.04	1.24 ± 0.08	4.16 ± 0.52	3.79 ± 0.36
0.10	1.33 ± 0.06	1.39 ± 0.04	3.92 ± 0.23	3.56 ± 0.18
TLCK (mM)				
0.01	0.01 ± 0.02	0.04 ± 0.02	0.14 ± 0.03	0.22 ± 0.02
0.10	0.01 ± 0.01	0.04 ± 0.01	0.08 ± 0.04	0.15 ± 0.02
TPCK (mM)				
0.10	1.11 ± 0.21	1.10 ± 0.20	3.13 ± 0.15	2.59 ± 0.12
1.00	0.00 ± 0.00	0.06 ± 0.01	0.00 ± 0.00	0.05 ± 0.02

same strong inhibitory effects without changing the survival rate of cells (99.7 and 99.8%, respectively vs 99.7% of control) measured by trypan blue exclusion method. PMSF showed moderate inhibitory effects on the release of both receptors. But the stronger effect observed for 4 mM might be secondary to lower survival rate of cells (76.9 vs 99.7% of control).

Effects of PMSF on the shedding of soluble TNF/LT receptors from the paraformaldehyde-fixed THP-1 cells by conditioned media

To further study whether the soluble TNF/LT receptors can be shed directly from the cell surface by proteases that can be inhibited by PMSF, THP-1 cells were fixed with paraformaldehyde and coincubated with conditioned media from 24 h PMA-stimulated THP-1 cells for 3 to 4 h. The amount of soluble TNF/LT receptors in the presence of PMSF is significantly lower than that of control and that in the presence of ethanol (Fig. 3*A* and *B*, $p < 0.001$ for both receptors). PMSF alone can slow down the degradation of soluble TNF/LT receptors when incubated with the conditioned media in the absence of paraformaldehyde-fixed cells (data not shown).

Effect of colchicine on the release of soluble TNF/LT receptors

Colchicine, an inhibitor of microtubule formation, stimulated the release of both soluble receptors (Fig. 4). The strength of induction appeared to be inversely related to dose. There was no cooperative effect between colchicine and PMA. The levels of soluble receptors were between the levels achieved with the individual agents used independently (Fig. 4). It seems that the inducing effect of PMA is antagonized by the colchicine.

Discussion

The soluble forms of membrane receptors have been identified for cytokine receptors IL-1R (21), IL-2R (22), IL-4R (23), and TNFR (7–10); receptors on lymphoid cells CD14 (24), CD23 (25), CD16 (26, 27), lymphocytes homing receptors (28), and CD27 (29); and receptors on nonlymphoid cells DAF (30) and GHR (31). A number of these soluble receptors have been identified in biologic fluids and they are still capable of specific ligand binding. The biologic significance of these soluble form receptors is under active investigation. It has been reported that alternative mRNA splicing is probably involved in producing a soluble IL-4R in mouse T cell line in vitro (23). Proteolysis and shedding were proposed as mechanisms involved in the release of several other soluble receptors (21, 22, 24–28).

Soluble form receptor derived from the 55 kDa TNF/LT receptor was identified by Gatanaga et al. (9) in the sera of cancer patients with various forms of cancer. This soluble form of 30 kDa has the ability to inhibit human TNF and LT activity both in vitro and in vivo (11). Elevated serum levels of soluble TNF/LT receptors in patients with solid tumors have also been reported by Aderka et al. (10). The ability of these receptors to inhibit TNF and LT bioactivity and the presence of the soluble TNF/LT receptors in these patients led these investigators to the hypothesis that soluble TNF/LT receptors may be immunosuppressive and allow cancer cells to evade host anti-tumor mechanisms. However, it was also proposed by Aderka et al. (12) that the soluble receptors at low receptor: TNF ratios could stabilize TNF molecules in vitro. It is clear that additional research is necessary to define the roles of these molecules in vivo.

In this study, soluble TNF/LT receptor release by a human macrophagelike cell line THP-1 was examined. These

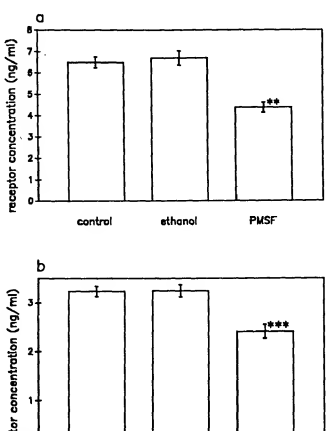

FIGURE 3. PMSF inhibits the shedding of soluble TNF receptors from paraformaldehyde-fixed THP-1 cells. The conditioned medium from PMA-stimulated THP-1 cells was preincubated with PMSF (2 mM) and ethanol (2%) for 1 h at 37°C. The paraformaldehyde-fixed THP-1 cells were then coincubated with the conditioned medium in the presence of ethanol (2%) or PMSF (2 mM) for 4 h at 37°C. The amount of the soluble receptors were assayed by ELISA. *A*: the concentration of soluble form of 55 kDa TNF/LT receptors. Control, 6.49 ± 0.26 ng/ml; ethanol, 6.69 ± 0.34 ng/ml; PMSF, 4.38 ± 0.24 ng/ml. *B*: the concentration of soluble form of 75 kDa TNF/LT receptor. Control, 3.23 ± 0.11 ng/ml; ethanol, 3.25 ± 0.12 ng/ml; PMSF, 2.41 ± 0.14 ng/ml. $***p < 0.001$.

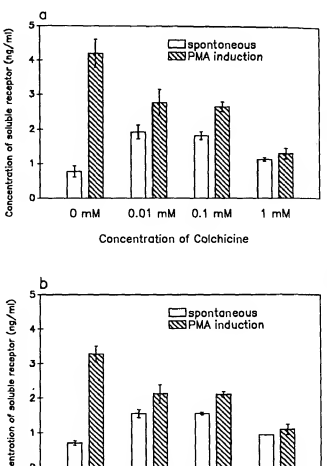

FIGURE 4. Colchicine stimulates the release of soluble TNF/LT receptors and inhibits the inducing effects of PMA. THP-1 cells were resuspended to 1×10^6 cells/ml in fresh medium and incubated with colchicine and/or PMA (10^{-8} M) for 8 h. The blank box shows effect of the colchicine on the spontaneous release. The hatched box shows effect of the colchicine on PMA-induced release. *A*: soluble form of 55 kD TNF/LT receptor. *B*: soluble form of 75 kDa TNF/LT receptor.

cells were stimulated with PMA, a protein kinase C activator, which was shown to stimulate the release of soluble forms of CD14 from human peripheral blood monocytes (24), homing receptors from mouse lymphocytes (28), and TNF/LT receptors from various human macrophage cell lines (15, 32, 33). The soluble TNF/LT receptors are not products of degradation because 1) they produce specific bands by Western blotting and 2) supernatants collected from frozen and thawed THP-1 cells destroys more than 70% of the soluble TNF/LT receptors preexisting in cell-free supernatants within 1 h of coincubation (data not shown). Although both TNF receptors showed almost the same response to PMA (Fig. 1), it cannot be concluded that the effect of PMA is nonspecific. It was shown that PMA did not stimulate the release of CD11c, MHC class I molecules, and CD64 (24). PMA-induced release of both soluble TNF/LT receptors is inhibited by all the serine proteases used in this study in a dose-dependent pattern. Spontaneous release is less sensitive to the inhibitory effect of serine proteases. This difference is best exemplified by 3,4 dichloroisocoumarin, which does not inhibit spontaneous release, whereas maintaining fairly strong inhibition of PMA-induced release. One possible explanation for this discrepancy is that PMA induction recruits serine proteases different from those responsible for the spontaneous release to increase the rate of soluble TNF/LT receptors production. These newly recruited serine proteases are relatively more sensitive to 3,4 dichloroisocoumarin and TPCK. The location where serine proteases work is most likely outside

the cell because PMSF significantly inhibits the production of soluble TNF/LT receptors from paraformaldehyde-fixed THP-1 cells. The ability of colchicine to counteract the inducing effect of the PMA also implies that PMA is recruiting serine proteases from inside the cells, possibly those stored in microtubule-associated vesicles. The speculated explanation for colchicine-induced receptor release is colchicine acting to inhibit internalization of TNF/LT receptors for turnover by lysosomes. The end result would be more TNF/LT receptors remaining on the plasma membrane for serine proteases to cut.

Serine proteases were found to be involved in the enzymatic cleavage and shedding of IL-1R from a human B cell line (21) and of CD14 from human monocytes in vitro (24). The release of the soluble CD16-II from human NK cells (34) and folate receptor from human nasopharyngeal carcinoma cells (35) was attributed to metalloproteases. Receptor shedding by proteases may represent a new mechanism in the turnover of membrane receptors and in the regulation of cell responsiveness to a ligand.

This report demonstrates that serine protease is responsible for both spontaneous and PMA-induced shedding of soluble forms of both TNF/LT receptors from the THP-1 cells in vitro. It supports the idea that the soluble receptor derived from the 55 kDa TNF/LT receptor is generated by extracellular proteolytic cleavage, as proposed by Lantz et al. (13). It further shows that the soluble form of the 75 kDa TNF/LT receptor is probably produced in the same manner.

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Thanks a lot...

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Mechanisms Involved in the Processing of the p55 and the p75 Tumor Necrosis Factor (TNF) Receptors to Soluble Receptor Forms

FLEMMING BJÖRNBERG, MIKAEL LANTZ, INGE OLSSON, and URBAN GULLBERG

ABSTRACT

The two tumor necrosis factor (TNF) receptors (TNF-R55 and TNF-R75) can release soluble TNF-binding proteins (TNF-R55-BP and TNF-R75-BP) by proteolytic cleavage. The proteolytic processing of the TNF receptors was investigated in monoblastic THP-1 and promyelocytic HL-60-10 leukemic cell lines. The release of soluble forms of both receptors was rapidly stimulated by staurosporine-sensitive protein kinase C activation by phorbol myristate acetate (PMA) and more slowly stimulated by TNF. No receptor release was seen below a temperature of 16°C. NH_4Cl (10 mmol/liter) and monensin (1 μmol/liter), known to increase intracellular pH, inhibited to some extent PMA- and TNF-induced release of both TNF-R55-BP and TNF-R75-BP. The inhibitory effect of monensin might be explained by a diminished translocation of newly synthesized receptor to the plasma membrane. The weak inhibitory effect of NH_4Cl on PMA-induced release of soluble receptor forms could be due to effects on a pH-sensitive compartment. PMA-induced down-regulation of receptors was not dependent on acidity as it occurred also in the presence of monensin and NH_4Cl when the release of TNF-BPs is partially blocked. Dibutyryl cAMP inhibited the PMA-induced release of TNF-R55-BP but not of TNF-R75-BP in both cell lines investigated. In addition, dibutyryl cAMP alone stimulated the release of both receptors but only in THP-1 cells. Our data show that the generation of soluble forms of both TNF receptors can be regulated by both PKC and PKA.

INTRODUCTION

Tumor necrosis factor (TNF-α; cachectin) and lymphotoxin (TNF-β) are homologous proteins with pleiotropic effects on cells (1,2). TNF seems to possess beneficial effects at low (physiological) concentration while high (pathological) concentration is associated with harmful manifestations. The latter is observed in the septic syndrome characterized by increased vascular permeability, hypotension, disseminated coagulation, and multiple organ failure and is mediated by cytokines such as TNF and interleukin-1 (IL-1) rather than by the microorganisms directly (3,4). Furthermore an association between the severity of meningococcal disease and serum levels of TNF has been reported (5). Cytokine inhibitors would therefore provide a rational therapy when cytokines are produced in excess leading to organ failure. The finding of TNF inhibitors in biological fluids consisting of TNF-binding proteins (TNF-BPs) (6–10) was therefore of importance. TNF-BPs represent soluble forms of transmembrane p55 (TNF-R55) or p75 (TNF-R75) receptors (11–15) produced by proteolytic cleavage of intact receptors.

Soluble forms exist for many cytokine receptors. These can be produced by proteolytic cleavage or be secreted forms derived from specific transcripts formed by alternative splicing of mRNA. A common occurrence of soluble receptor forms suggests that they have important biological functions (16). The latter could involve regulation of systemic and local effects of cytokines as well as slow release of ligand from a complex between soluble receptor and cytokine to provide physiological concentrations of cytokines in tissues. Soluble receptor forms could also dissociate and bind cytokines that are immobilized by binding to proteoglycans of extracellular matrix (17) followed by transportation and/or elimination.

The proteases that catalyze the cleavage of the TNF receptors to generate TNF-BPs regulate the number of receptors on cells and the release of TNF-BPs, thereby modulating the action of TNF. Therefore, these proteases are potential therapeutic tar-

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gets in conditions such as the septic syndrome, graft-versus-host disease, and chronic inflammatory disorders. Activation of protein kinase C (PKC) causes a rapid decrease in the cellular binding of TNF due to a reduction of the number of binding sites (18–20). Moreover, down-regulation of TNF receptors by activation of protein kinase C increases the production of TNF-BPs (21). Thus, it is likely that PKC activates proteases that cleavage the receptors with the subsequent release of TNF-BPs. In the present work we have investigated the proteolytic processing of the TNF receptors in leukemia cell lines.

MATERIALS AND METHODS

Reagents

Phorbol 12-myristate 13-acetate (PMA), dibutyryl cyclic adenosine monophosphate (dbcAMP), NH_4Cl, monensin, and sodium butyrate were from Sigma Chemicals, St. Louis, MO. Recombinant TNF (produced by Genentech Inc., San Francisco, CA) and recombinant TNF-R55-BP were kindly provided by Dr. G. Adolf, Bender+Co, Vienna, Austria. Recombinant TNF-R75-BP was a gift from Dr. H. Gallati, Hoffmann-LaRoche, Basel, Switzerland. Staurosporine was from Boehringer Mannheim Scandinavia AB, Bromma, Sweden.

Antibodies

Polyclonal antiserum against TNF-R55-BP was produced in rabbits as previously described (8,17,21) and has been characterized by use in immunoassays and immunoprecipitation studies (17,21). Monoclonal antibodies to TNF-R55-BP (TBP-1, TBP-2) were kindly provided by Dr. G. Adolf, Bender+Co, Vienna Austria. A monoclonal antibody to TNF-R75-BP (utr-4) was a generous gift from Dr. H. Gallati, Hoffman-LaRoche, Basel, Switzerland. A rat monoclonal antibody to TNF-R75 was purchased from Genzyme, Cambridge, MA.

Cell culture

The following cell lines were used: HeLa, monoblastic THP-1, monoblastic U-937, and promyelocytic HL-60. A subclone of HL-60 (HL-60-10) was used because of its higher production of TNF-BPs compared to wild type. Cell lines were maintained in suspension culture in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). Experiments with TNF-BP release were carried out with 2×10^6 cells in 1 ml growth medium in 24-well tissue culture plates or in constantly rotating micro tubes when using HL-60-10 and THP-1 cells, respectively. At the end of the incubation cells were pelleted by centrifugation and supernatants were collected and stored at –20°C until analyzed for TNF-R55-BP and TNF-R75-BP with ELISA. In experiments with NH_4Cl, monensin, or staurosporine, cells were preincubated with these substances for 15 min prior to induction of TNF-BP release and then present throughout the experiment.

Receptor binding studies

Iodination of TNF (10 µg) was carried out with 1 mCi ^{125}I-NaI (Amersham, Amersham, U.K.) using Iodobeads® (Pierce, Oud Beijerland, The Netherlands) according to the manufacturer's description. Specific binding of TNF to cells was determined as described (22). All steps were performed at 4°C. Briefly, 4×10^6 cells were incubated with ^{125}I-TNF in 200 µl binding buffer consisting of Dulbecco's phosphate-buffered saline (PBS), pH 7.4 and 1% bovine serum albumin (BSA) under gentle rotation at 4°C for 2 h. After incubation the cells were washed quickly twice with cold binding buffer. The radioactivity of the cell pellet was determined by use of a gamma-counter. Nonspecific binding was determined in the presence of a 100-fold excess of unlabeled TNF and was typically 5–10%. By incubation with 2.5 µg/ml neutralizing antibody specific for TNF-R55-BP (TBP-2) or TNF-R75 (Genzyme Cambridge, MA) during the assay, binding to either receptor was determined in the following way: Specific binding to both receptors (combined specific binding) was defined as total binding minus unspecific binding that occurred in the presence of unlabeled TNF as described above. Specific binding to TNF-R55 was defined as specific binding that occurred in the presence of anti-TNF-R75. Similarly, specific binding to TNF-R75 was determined in the presence of anti-TNF-R55.

Determination of TNF-R55-BP and TNF-R75-BP with enzyme-linked immunosorbent assay (ELISA)

A sandwich ELISA was used for determination of TNF-R55-BP as previously described (17) and an assay for TNF-R75-BP was developed by the same principle. Immunoplates were coated for at least 3 h with a monoclonal antibody (TBP-1) to TNF-R55-BP (23), 2.5 µg/ml, or a monoclonal antibody (utr-4) (24) to TNF-R75-BP, 5 µg/ml. Plates coated with utr-4 were further incubated with 1 mg/ml BSA for 3 h at 37°C for blocking of unspecific binding sites. Samples were loaded in duplicate and incubated over night at 4°C. After washing, a polyclonal antiserum to TNF-R55-BP (21) or a rat monoclonal antibody to TNF-R75-BP (Genzyme, Cambridge, MA), 0.2 µg/ml, was added and plates were incubated for 3 h at 20°C and washed. A peroxidase-conjugated goat anti-rabbit antibody (Bio-Rad, Hercules, CA) or a peroxidase-conjugated goat anti-rat IgG_{2b} (Kemila, Sollentuna, Sweden), diluted 1:2500, was allowed to bind during incubation for 1 h at 20°C. After washing, tetramethyl benzidine (Bio-Rad, Hercules, CA) was added as substrate and absorbance was measured at 660 nm or, after addition of H_2SO_4, at 450 nm in a Titertek multiscan ELISA plate reader. Values were calculated from a standard curve based on freshly prepared dilutions, 0.03–3 ng/ml recombinant TNF-R55-BP, or 0.08–1.25 ng/ml recombinant TNF-R75-BP. The detection limit for TNF-R55-BP was 0.03 ng/ml and for TNF-R75-BP 0.08 ng/ml.

Biosynthetic labeling of cells

HL-60 or HeLa cells (4×10^7) were depleted of cysteine by incubation in cysteine-free Eagle's minimal essential medium (Flow laboratories) for 30 min at a concentration of 2×10^6/ml followed by incubation for 30 min at a concentration of 4×10^6/ml cysteine-free Eagle's minimal essential medium containing 10% dialyzed FBS and 165 µCi/ml [^{35}S]cysteine (specific activity more than 1000 Ci/mmol) for biosynthetic labeling of cell proteins. This was followed by chase of the label for various periods of time in complete medium with 10% FBS at a

cell concentration of 2×10^6/ml. Cells were lysed in 1 ml cold radioimmunoprecipitation assay (RIPA) buffer consisting of 0.15 mol/liter NaCl, 30 mmol/liter Hepes (pH 7.3), 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and freshly added PMSF at 1 mmol/liter. After 1 h, cell lysates were clarified by centrifugation at 32,000*g* for 30 min at 4°C. Cell supernatants were lyophilized, resuspended in 1 ml RIPA buffer, and dialyzed against RIPA buffer. RIPA buffer extracts, 0.5–1.5 ml, of whole cells or cell supernatant were mixed with antibody and 50 μl protein A-Sepharose suspension (200 mg/ml) and incubated overnight at 4°C under continuous rotation. SDS-PAGE was then performed on 16% acrylamide slab gels as described (25). After electrophoresis the gels were stained, destained, incubated with Amplify (Amersham, Amersham, UK), and dried on filter paper. Dried gels were exposed to X-ray film (Hyperfilm MP, Amersham, Amersham, UK) at –80°C for 1 week. Apparent molecular weight values were determined by use of molecular weight standards (Pharmacia, Uppsala, Sweden), which included phosphorylase b, 94,000; bovine serum albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor, 20,100; and lactalbumin, 14,400.

RESULTS

Biosynthesis of the TNF-R55-BP

The biosynthesis of both TNF-R55 and TNF-R55-BP has been demonstrated previously by biosynthetic labeling followed by immunoprecipitation with a polyclonal antiserum against TNF-R55-BP (21). Figure 1 demonstrates that the monoclonal antibodies TBP-1 and TBP-2 (23) both recognized TNF-R55-BP in cell supernatant. Contrary to TBP-2, TBP-1 failed to recognize intact TNF-R55 in cell lysate (Fig. 1). The lack of recognition by TBP-1 is most likely explained by the appearance of a novel epitope on the soluble form, absent on intact TNF-R55, against which TBP-1 is directed. ^{125}I-labeled TBP-1 showed no specific binding to the cell surface (data not shown), which is consistent with the lack of binding of this antibody to intact TNF-R55 in cell lysates. Binding of labeled TBP-2 to cells was inhibited by TNF, lymphotoxin, unlabeled antibodies, or recombinant TNF-R55-BP indicating specific binding to TNF-R55 (data not shown).

Production of TNF-R55-BP and TNF-R75-BP, effects of monensin and NH_4Cl

The release of TNF-R55-BP and TNF-R75-BP was shown to be stimulated by both PMA and TNF in the cells investigated (Fig. 2). It should be emphasized, however, that the effect of TNF was considerably delayed compared to that of PMA. The temperature dependency of PMA-induced cleavage of the receptors was also investigated (Fig. 3). Almost no receptor cleavage was seen below a temperature of 16°C. The effects of monensin and NH_4Cl, known to increase intracellular pH, are shown in Figs. 4 and 5. To simplify a comparison of the results, the data have been normalized and expressed as percentage of maximal release induced by PMA or TNF alone. The detection limit of the assay for TNF-R55-BP and TNF-R75-BP, corresponding to 0.03 and 0.08 ng/ml, respectively, is indicated in each figure. NH_4Cl (10 mmol/liter) and monensin (1 μmol/liter) alone did not promote release of TNF-BPs above the detection limit (data not shown). Both monensin and NH_4Cl inhibited PMA- and TNF-induced release of TNF-R55-BP and TNF-R75-BP to a variable degree in both cell lines investigated (Figs. 4 and 5). The inhibition by 10 mmol/liter NH_4Cl was generally weak and variable while that of 1 μmol/liter monensin was stronger in most cases. The effects were similar on both receptor forms. Cells incubated with monensin and NH_4Cl still responded to PMA with total down-regulation of TNF receptors, measured as inhibition of binding of TNF (Fig. 6). Thus, down-regulation of these receptors with PMA is not dependent on acidity and is unaffected when the release of TNF-BPs is inhibited.

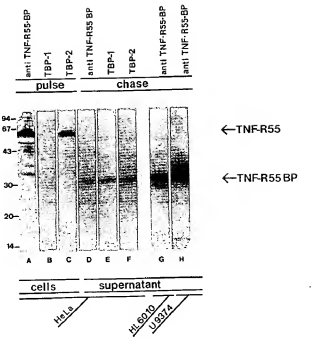

FIG. 1. Immunoprecipitation of TNF-R55 and TNF-R55-BP. Upper part of the figure. HeLa (lanes A–F), HL-60-10 (lane G), and U-937-4 (lane H) cells were labeled with [^{35}S]cysteine for 30 min and the label was chased for zero minutes (lanes A–C) or 120 min in the presence of 10 ng/ml PMA (lanes D–H). Cells (lanes A–C) and cell supernatants (lanes D–H) were recovered and immunoprecipitated with 1 μl polyclonal anti-TNF-R55-BP (lanes A, D, G, H), 1 μg monoclonal TBP-1 (lanes B, E) and 1 μg monoclonal TBP-2 lanes (C, F). Molecular weight markers are indicated and TNF-R55 and TNF-R55-BP are indicated with arrows.

The inhibitory effect of monensin and NH_4Cl on the PMA-induced release of soluble receptor forms could be due to elevation of pH and/or inhibition of translocation of new receptor to the plasma membrane. In the latter case monensin and NH_4Cl alone are expected to decrease the number of TNF receptors. Therefore the effects with time of these agents on TNF binding to the cell surface were determined (Fig. 7). Monensin strongly decreased TNF binding, indicating that its inhibition of TNF-BP release could be due to an effect on receptor replenishment. The effect of NH_4Cl on TNF binding was negligible during the first 4 h of incubation, while a significant effect was seen after 16–24 h of incubation. Thus the effects of NH_4Cl on the slow

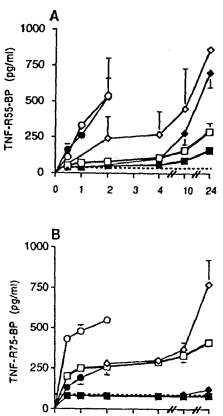

FIG. 2. PMA- and TNF-induced release TNF-R55-BP and TNF-R75-BP. THP-1 (open symbols) and HL-60-10 cells (filled symbols) were incubated for various time periods with 10 ng/ml PMA (●,○), 1 nmol/liter TNF (♦,◇), and without additions (■,□). Cell supernatants were collected and analyzed for TNF-R55-BP (A) and TNF-R75-BP (B) as described in Materials and Methods. Bars represent SEM ($n = 5$) and bars not shown fall within the limit of the symbols. Broken line indicates the detection limit of the assay.

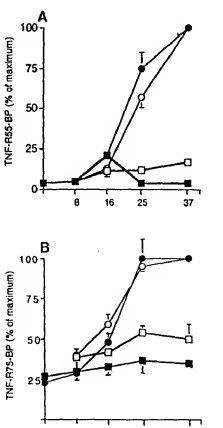

FIG. 3. Release of TNF-R55-BP and TNF-R75-BP is temperature dependent. THP-1 (open symbols) and HL-60-10 cells (filled symbols) were incubated for 2 h at different temperatures with 10 ng/ml PMA (●,○) and without additions (■,□). Cell supernatants were collected and analyzed for TNR-R55-BP (A) and TNF-R75-BP (B). Results are normalized and expressed as percentage of maximal release induced by PMA at 37°C. Bars represent SEM ($n = 3$) and bars not shown fall within the limit of the symbols.

TNF-induced release of TNF-BP might be explained by effects on receptor translocation. On the other hand, the inhibition of the rapid PMA-induced release of TNF-BP might be due to changes in pH.

Roles for both PKC and PKA in the production of TNF-R55-BP and TNF-R75-BP

Down-regulation of the TNF receptors may be the result from proteolytic cleavage and/or from internalization of receptors. As shown above, incubation of cells with PMA resulted in down-regulation of TNF receptors with production of soluble forms. Both these events were blocked by the protein kinase C (PKC) inhibitor staurosporine (Figs. 6 and 8) indicating a role for PKC. Thus neither cell surface proteolytic processing nor internalization of receptors occurs in the presence of staurosporine.

The PMA-induced release of TNF-R55-BP was inhibited by the cAMP raising agent dbcAMP (1 mmol/liter), known to activate protein kinase A (PKA), both in HL-60-10 and THP-1 cells (Fig. 9). However, dbcAMP alone induced release of TNF-R55-BP in THP-1 cells but not in HL-60-10 cells. dbcAMP alone also induced release of TNF-R75-BP in both THP-1 and HL-60-10 cells. The effect on HL-60-10 cells was slow and visible first after 12–24 h when release similar to that seen with PMA was seen (not shown in Fig. 9). Contrary to results for TNF-R55-BP, dbcAMP did not inhibit PMA-induced release of TNF-R75-BP. As dbcAMP is metabolized to cAMP and butyrate within the cell, controls were performed with 1 mmol/liter sodium butyrate, which did not affect release of TNF-BPs either alone or in combination with PMA (data not shown).

DISCUSSION

Membrane-bound proteins also exist as soluble isoforms being released by limited proteolytic cleavage of an extracellular domain (16). Among such proteins are cytokine receptors

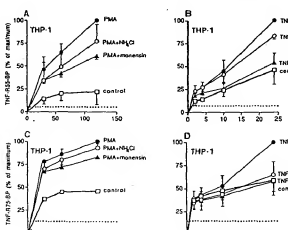

FIG. 4. Modulation of PMA- and TNF-induced release of TNF-R55-BP and TNF-R75-BP from THP-1 cells. (A,C): THP-1 cells were incubated for various time periods with 10 ng/ml PMA alone (●) and in combination with 10 mmol/liter NH_4Cl (○) or 1 μmol/liter monensin (▲). (B,D) THP-1 cells were incubated for varius times with 1 nmol/liter TNF alone (●) and in combination with 10 mmol/liter NH_4Cl (○) or 1 μmol/liter monensin (▲). Cells were preincubated for 15 min with NH_4Cl and monensin before addition of PMA or TNF. Control incubations were performed without additions (□). Cell supernatants were collected and analyzed for TNF-R55-BP (A,B) and TNF-R75-BP (C,D). Results were normalized and expressed as percentage of maximal release induced by PMA or TNF alone. Bars represent SEM ($n = 5$) and bars not shown fall within the limit of the symbols. Broken line indicates the detection limit of the assay.

where cleavage leads to both desensitization of cells and release of soluble inhibitor of the cytokine. After attachment of neutrophils to endothelial cells, the adhesion molecule L-selectin is cleaved as a prerequisite for transmigration of neutrophils (26). Membrane-anchored transforming growth factor-α (TGF-α) is selectively cleaved to generate a soluble form (27). None of the proteases involved in the release of transmembrane proteins by limited proteolysis has been identified. The present work provides some characterization of the proteolytic events leading to generation of soluble TNF receptor forms. We demonstrate that phorbol ester-induced PKC activation gives rise to a rapid cleavage of the receptors with release of TNF-BPs while incubation with TNF results in a slow production of TNF-BPs from some cell types.

The carboxyl terminus of TNF-R55-BP purified from urine consists of Asn_{172} (13). Asn_{172} and Val_{173} are the only amino acids in this region conserved in man, mouse, and rat TNF-R55 sequences (13,28). This may suggest that a protease can catalyze a cleavage between Asn_{172} and Val_{173}. Site-directed mutagenesis or deletions of amino acids in this region leads to diminished release of TNF-R55-BP (29,30). However, a minor portion of purified TNF-R55-BP with the carboxyl terminus extended beyond Asn_{172} has been reported (30). An initial cleavage could therefore occur closer to the plasma membrane followed by further extracellular proteolysis to generate the mature TNF-R55-BP detectable in most biological fluids. Alternatively, distinct carboxyl termini of TNF-BP may reflect different proteolyic processing mechanisms of transmembrane receptor. Hypothetically, such diverse processing could be tissue specific or dependent on the stimulation for receptor shedding.

By deletion of the intracellular domain of TNF-R55 the signaling capacity was found to be abolished but neither spontaneous nor phorbol ester-induced generation of TNF-R55-BP seemed to be affected (31). A deletion mutant of TNF-R75, lacking the entire cytoplasmic part of the receptor, could be induced to down-regulation and shedding, although poorly as compared to full length receptor (32), suggesting that the cytoplasmic domain of TNF-R75 is important but not essential for spontaneous or PMA-induced cleavage of TNF-R75. Thus PKC-induced receptor cleavage of TNF receptors does not seem to be dependent on phosphorylation of the receptor, rather a protease or a cofactor may be activated by phosphorylation as a prerequisite for cleavage. In contrast to TNF-R55, membrane-anchored TGF-α (proTGFα) required an intact carboxyl-terminal of the cytoplasmic tail for generation of soluble growth factor by proteolytic cleavage (27). In the latter case an inside-out transfer of information across the membrane is therefore implied for controlling a specialized proteolytic system that acts close to the cell surface.

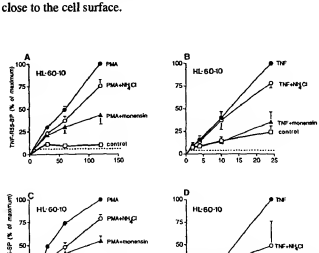

FIG. 5. Modulation of PMA- and TNF-induced release of TNF-R55-BP and TNF-R75-BP from HL-60 cells. (A,C) HL-60-10 cells were incubated for various time periods with 10 ng/ml PMA alone (●) and in combination with 10 mmol/liter NH_4Cl (○) or 1 μmol/liter monensin (▲). (B,D) HL-60-10 cells were incubated for various times with 1 nmol/liter TNF alone (●) and in combination with 10 mmol/liter NH_4Cl (○) or 1 μmol/liter monensin (▲). Cells were preincubated for 15 min with NH_4Cl and monensin before addition of PMA or TNF. Control incubations were performed without additions (□). Cell supernatants were collected and analyzed for TNF-R55-BP (A,B) and TNF-R75-BP (C,D). Results are normalized and expressed as percentage of maximal release induced by PMA or TNF alone. Bars represent SEM ($n = 5$) and bars not shown fall within the limit of the symbols. Broken line indicates the detection limit of the assay.

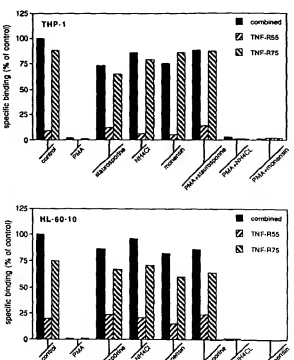

FIG. 6. Modulation of TNF-binding to TNF-R55 and TNF-R75. THP-1 and HL-60-10 cells were incubated with 10 ng/ml PMA for 1 h at 37°C whereafter specific binding to TNF-R55 and TNF-R75 or to both receptors (combined) was determined. In addition, cells were preincubated for 15 min with 1 μmol/liter staurosporine, 10 mmol/liter NH_4Cl, or 1 μmol/liter monensin prior to incubation with PMA. Control incubations were performed without additions. Combined specific binding of control cells was 1565 cpm (HL-60-10) and 731 cpm (THP-1). One representative experiment is shown.

TNF receptor down-regulation can take place either by receptor cleavage under generation of TNF-BP's or by receptor internalization. The effects on receptor down-regulation of a weak base amine, NH_4Cl, and of a monovalent carboxylic ionophore, monensin, were investigated. Neither NH_4Cl nor monensin affected PMA-induced down-regulation of TNF-receptors. These agents can inhibit proteolysis by raising pH in endosomes and lysosomes (33). Thus the inhibitory effect by NH_4Cl and monensin on PMA-induced release of TNF-BP suggests that endosomal processing could be involved in the generation of soluble receptor forms. NH_4Cl did not affect TNF binding during short-time incubation, ruling out an inhibition of translocation of new receptor to the cell surface. However, due to the fact that only a weak inhibitory effect was observed on the release of TNF-BP, it is not possible to definitely conclude that NH_4Cl inhibited receptor cleavage by an increase in pH. Monensin, on the other hand, clearly interferes with receptor translocation as judged by diminished TNF binding in control experiments. The release of soluble forms of TNF receptor upon incubation with PMA is a rapid event, consistent with cleavage taking place on the plasma membrane. Recently, a proteolytic processing of TNF receptors from the surface of THP-1 cells involving serine-proteases has been reported both for spontaneous and PMA-induced shedding of soluble receptor forms (34).

In addition to PMA, TNF also induced release of TNF-BPs from both receptors, which was inhibited by NH_4Cl and monensin. As the effects of TNF in this case are slow, the mechanisms by which NH_4Cl and monensin inhibit release of soluble receptor forms may be dependent on interference with translocation of new receptor to the plasma membrane. PMA- and TNF-induced release of TNF-BPs may involve similar cellular mechanisms. It is important to consider that TNF-induced activation of PKC could be cell specific because it has been reported previously not to affect shedding of TNF-R75 from a cell line overexpressing this receptor (35). Moreover, incubation of HeLa cells or human umbilical vein endothelial cells (HUVEC) with TNF did not result in increased production of TNF-BP (data not shown). Our results are, however, consistent

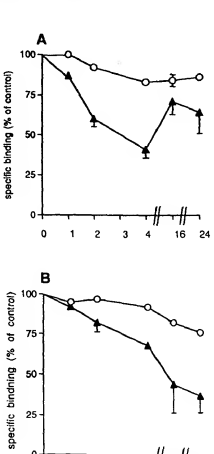

FIG. 7. Effects of NH_4Cl and monensin on TNF-binding. THP-1 (A) and HL-60-10 (B) cells were incubated without (control cells) or with 10 mmol/liter NH_4Cl (○) or 1 μmol/liter monensin (▲) at 37°C for 1, 2, 4, 16, and 24 h whereafter specific binding of TNF was determined. Percentage of combined specific binding of TNF as compared to control cells is shown. Bars represent SEM ($n = 3$) and bars not shown fall within the limit of the symbols. Specific binding to control cells was 4556 ± 842 (SEM) cpm (HL-60-10) and 2990 ± 640 (SEM) cpm (THP-1).

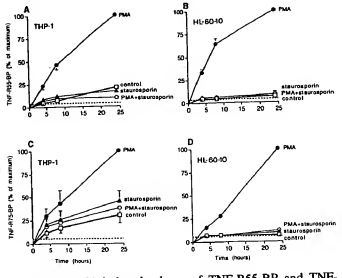

FIG. 8. PMA-induced release of TNF-R55-BP and TNF-R75-BP involves PKC. THP-1 (A,C) and HL-60-10 cells (B,D) were incubated with 10 ng/ml PMA (●), 1 μmol/liter staurosporin (▲), and PMA plus staurosporine (○) for various time periods. Control incubations were performed without additions (□). Cell supernatants were collected and analyzed for TNF-R55-BP (A,B) and TNF-R75-BP (C,D). Results are normalized and expressed as percentage of maximal release induced by PMA alone. Bars represent SEM ($n = 3$) and bars not shown fall within the limit of the symbols. Broken line indicates the detection limit of the assay.

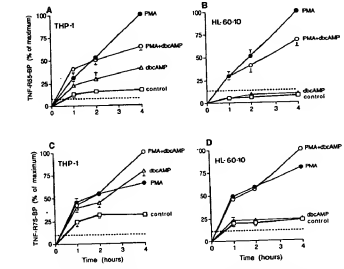

FIG. 9. Modulation of PMA-induced release of TNF-R55-BP and TNF-R75-BP by cAMP. THP-1 (A,C) and HL-60-10 cells (B,D) were incubated with 10 ng/ml PMA (●), 1 mmol/liter dbcAMP (△), and PMA plus dbcAMP (○) for various time periods. Control incubations were performed without additions (□). Cell supernatants were collected and analyzed for TNF-R55-BP (A,B) and TNF-R75-B0 (C,D). Results are normalized and expressed as percentage of maximal release induced by PMA alone. Bars represent SEM ($n = 3$) and bars not shown fall within the limit of the symbols. Broken line indicates the detection limit of the assay.

with results from incubation of THP-1 cells when LPS-induced TNF production was reported to release TNF-BPs (36). In addition, TNF can promote the release of TNF-BP *in vivo* as infusion of TNF leads to a rapid but transient increase of serum TNF-BP in patients with cancer (37). Similar mechanisms may therefore operate both *in vitro* and *in vivo* to produce TNF-BPs.

The ratio of TNF-R55 and TNF-R75 differs on various cells. Therefore the two receptors might be regulated differently in a cell-specific manner. It has been shown that cAMP can regulate the expression of TNF receptors (22,38,39). Some results indicate that PKA andf PKC antagonistically regulate both TNF production and, at the receptor level, TNF sensitivity (38). The effect of PKA on receptor levels has, on the basis of cross-linking experiments and TNF-binding studies, been reported to be restricted to up-regulation of TNF-R75 and not TNF-R55 (22,39). Thus dbcAMP induced a 3- to 6-fold increase in the number of TNF-R75 in HL-60 cells without affecting the number of TNF-R55. These results suggest that dbcAMP specifically up-regulates TNF-R75. The effect may be explained by our previous finding of a PKA-mediated transcriptional control selectivity for TNF-R75 (22). The present results show that dbcAMP not only up-regulates TNF-R75 but in addition enhances extracellular release of this receptor. The latter effect was not restricted to TNF-R75 inasmuch as a similar effect is seen for TNF-R55 in THP-1 cells. Moreover, as judged by results from the two cell lines investigated, dbcAMP specifically inhibited the PMA-induced release of TNF-R55-BP. In conclusion, it is demonstrated that the production of soluble forms of TNF-R55 and TNF-R75 may be independently regulated through mechanisms involving PKC and PKA. Furthermore, our results suggest that tissue-specific processes for receptor cleavage involving a pH-dependent component may exist.

ACKNOWLEDGMENTS

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Thanks a lot...

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Expression and cleavage of tumor necrosis factor-α and tumor necrosis factor receptors by human monocytic cell lines upon direct contact with stimulated T cells

Tumor necrosis factor-α (TNF-α) is a potent cytokine in inflammatory processes. A variety of mechanisms that modulate its activity have been described, one being its binding to soluble receptors (sTNFR). In this study, we demonstrate that human monocytic cells such as THP-1 respond to direct contact with a membrane preparation of stimulated HUT-78 cells by producing TNF-α and by releasing sTNFR-p75, but not sTNFR-p55, with different kinetics. TNF-α concentration peaked after 12 h of contact and then decreased, whereas sTNFR-p75 production increased progressively upon cell/cell contact. The decrease in TNF-α concentration is not due to trapping of TNF-α by its soluble receptors or other soluble or cell-associated molecules, but rather to a proteolytic activity associated to THP-1 cells. On the other hand, the increase in sTNFR-p75 release does not result from an increase in the cleavage of pre-existing cell-associated sTNFR-p75 but from an increase in TNFR-p75 expression, immediately followed by the cleavage of its extracellular domain. Phenylmethylsulfonylfluoride, a serine protease inhibitor, has a negative effect on both TNF-α degradation and sTNFR-p75 release by THP-1 cells. Thus, there may be an enzymatic activity associated to THP-1 cells that plays an important role in the neutralization of TNF-α activity both by degrading the molecule and by cleaving its receptors at the cell surface.

1 Introduction

TNF-α is a potent cytokine produced mainly by activated monocyte/macrophages, but also by other cells including B and T lymphocytes, NK cells, Kupffer cells, glial cells and adipocytes. Human TNF-α is translated as a transmembrane protein of 26 kDa which is cleaved by a metalloproteinase [1–3] to give rise to the 17 kDa soluble form of TNF-α. In solution, only the trimer of TNF-α is biologically active. TNF-α is a multifunctional cytokine involved in many biological processes. It is the first cytokine to be detected in serum in endotoxemia and bacteremia, reaching a peak at 90 min, while IL-1 displays a peak at 3–4 h and IL-6 levels rise throughout ≈ 8 h [4–6]. TNF-α is thought to play a pivotal role in inflammatory processes. During lethal bacteremia, infusion of anti-TNF-α monoclonal antibodies attenuates both IL-1 and IL-6 levels [6], suggesting that TNF-α is essential for initiating or amplifying IL-1 and IL-6 release during septic shock syndrome. Similarly, the capacity of whole peripheral blood cells from multiple sclerosis patients to produce TNF-α upon PHA stimulation is enhanced during 4–6 weeks preceding a relapse [7]. Furthermore, beneficial effects of TNF-α blockade have been demonstrated in rheumatoid arthritis [8]. The control of TNF-α production and activity in chronic inflammatory diseases is therefore the aim of numerous investigations.

TNF-α acts on target cells by binding to two transmembrane receptors of 55 kDa (type I) and 75 kDa (type II) which display different biochemical characteristics (for review, see [9]). Most cell types and tissues express both TNFR types which transduce distinct signals [9]. Interestingly, the two TNFR were identified after the isolation of TNF-binding proteins in urine from febrile patients or serum from cancer patients [10–12]. These binding proteins proved indeed to be soluble TNF receptors (sTNFR) [13–15]. To date, the two soluble receptors are the only natural inhibitors that interfere with TNF-α activity by impairing its interaction with the target cell. As in the case of TNF-α processing, the shedding of the extracellular domains of TNFR is secondary to cleavage by a metalloproteinase, unidentified as yet [16]. Since sTNFR display a similar affinity for TNF-α as membrane receptors, it is likely that *in vivo* they modulate, rather than inhibit the TNF-α signal. A 10- to 100-fold molar excess of sTNFR over TNF-α is required for complete inhibition of TNF-α activity [17]; their physiological levels in biological fluids are at least twofold higher than those of TNF-α. In monocyte/macrophages, TNF-α production is induced by a variety of stimuli, including LPS, tumor promoters, viruses and mitogens. Other cytokines that stimulate TNF-α production to a small extent include IFN-α, IL-1, and various CSF. A more powerful stimulation of TNF-α production by monocytes is provided by direct cell/cell contact with stimulated T lymphocytes [18]. Indeed, we have recently shown that this novel mechanism, *i.e.* direct

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Abbreviations: TNFR: Tumor necrosis factor receptor **sTNFR:** soluble TNFR

Key words: Tumor necrosis factor-α / THP-1 cell line / T cell / Cell contact

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cell/cell contact, might play a major role in inducing the production of pro-inflammatory factors on monocytes and monocytic cells [18, 19–22]. To shed light on the mechanisms which control TNF-α activity upon stimulation of monocytes by direct contact with stimulated T lymphocytes, we analyzed simultaneously the production of TNF-α and release of sTNFR.

2 Materials and methods

2.1 Media and reagents

Heat-inactivated FCS, streptomycin, penicillin, L-glutamine, RPMI, HBSS, Hepes and PBS were obtained from Gibco (Paisley, Scotland), paraformaldehyde from Merck (Darmstadt, Germany), purified PHA from Wellcome Diagnostics (Dartford, GB), PMSF, PMA, BSA, chloramine T, actinomycin D and cycloheximide from Sigma (St. Louis, MO). LPS was isolated from *Salmonella minnesota* strain Re595.

2.2 C ll lines

THP-1, a human myelomonocytic cell line [23], and HUT-78, a human T cell line [24], were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated FCS, 50 μg/ml streptomycin, 50 IU/ml penicillin and 2 mM L-glutamine (medium) in a 5% CO_2-air humidified atmosphere at 37°C. HUT-78 cells were stimulated by 1 μg/ml PHA and 5 ng/ml PMA in medium. After 24 h, cells were washed and counted; viability was >95% as assessed by trypan blue exclusion. HUT-78 plasma membranes were prepared as described [19]. Pelleted membranes were resuspended in medium so that 100 μl was equivalent to 4×10^5 HUT-78 cells.

2.3 Stimulation of monocytic cells by membrane preparation of HUT-78 cells

THP-1 cells were washed twice in PBS and resuspended in medium at 5×10^5 cells/ml. The cell suspension (100 μl) was dispensed onto a 96-well culture plate, some wells being supplemented with 100 μl of medium and others with 100 μl of HUT-78 plasma membranes. After different periods of culture in a 5% CO_2-air humidified atmosphere at 37°C, supernatants were analyzed for cytokine content. When required, inhibitors of signal transduction were added to monocytic cells for 30 min at 37°C prior to the addition of HUT-78 cell membranes.

2.4 Binding assay for ^{125}I-labeled TNF-α

TNF-α and IL-1β iodination by the chloramine T method [25] and binding of iodinated TNF-α on THP-1 cells was assessed as described [11].

2.5 Cytokine detection

Production of IL-1β was measured by ELISA as described [26] (EIA IL-1β kit, Immunotech, Luminy, France, sensitivity 10 pg/ml). Production of TNF-α was measured by an enzyme-amplified sensitivity immunoassay kit (EASIA kit, Medgenix, Fleurus, Belgium, sensitivity 15 pg/ml). For detection of radiolabeled TNF-α, Laemmli sample buffer [27] was added to the supernatants collected to yield a final concentration of 0.25% SDS, 0.01% bromophenol blue and 15 mM Tris-HCl pH 6.8. Samples were subjected to 15% polyacrylamide gel electrophoresis and analyzed by autoradiography of dried gels. sTNFR-p55 and sTNFR-p75 were detected by an enzyme-bound immunological biological assay (ELIBA, Hoffmann-La Roche, Basel, Switzerland, sensitivity 0.3 ng/ml).

3 Results

3.1 TNF-α production during contact between monocytic cells and membrane preparation of HUT-78 cells

To assess the influence of T cell surface factors in monocyte activation, we cultured THP-1 cells with membrane preparations of HUT-78 cells. As shown in Fig. 1 and confirmed by other experiments (data not shown), membranes of stimulated HUT-78 cells contained significant amounts of TNF-α which accounted for the high level of cytokine observed after 15 min. TNF-α production by THP-1 cells progressively increased between 1 and 12 h of contact and decreased between 12 and 72 h of contact. Membrane preparations of resting HUT-78 cells did not induce TNF-α production by THP-1 cells or release any TNF-α. This confirmed that stimulated HUT-78 cells express cell surface factors which trigger monocytic cells to produce TNF-α. To assess whether this response was specific for contact with HUT-78 cell membranes, LPS or PMA was added to THP-1 cells and TNF-α production was measured after different periods of time (Fig. 1). LPS was not able to induce a significant THP-1 cell response, whereas PMA induced a substantial production of TNF-α.

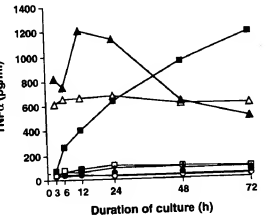

Figure 1. TNF-α production by THP-1 cells in response to membranes of stimulated HUT-78 cells, LPS or PMA. THP-1 were cultured in the absence (closed diamonds), or in the presence of membranes of resting (closed circles) or stimulated HUT-78 cells (closed triangles), 20 μg/ml LPS (open squares) or 5 ng/ml PMA (closed squares). As control, membranes of resting (open circles) or stimulated HUT-78 cells (open triangles) were cultured in the absence of THP-1 cells. Supernatants were collected at the indicated time and tested for TNF-α by ELISA. Initial values of TNF-α (open triangles) are due to endogenous TNF-α associated with stimulated HUT-78 cell membranes.

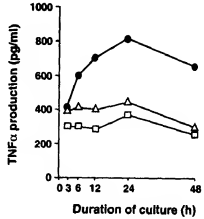

Figure 2. New transcription and new translation for TNF-α after 3 h of contact between THP-1 cells and membranes of stimulated HUT-78 cells. THP-1 cells were cultured with stimulated membranes in the absence (closed circles) or presence of 5 μg/ml cycloheximide (open triangles) or 5 μg/ml actinomycin D (open squares). Supernatants were collected at the indicated time to measure TNF-α production. Initial values of TNF-α are due to endogenous TNF-α associated with the stimulated HUT-78 cell membranes.

The possibility that the activation of THP-1 cells by membranes of stimulated HUT-78 cells was due to carryover of PHA, PMA or both was ruled out in a previous study [20].

To ascertain whether the increase in TNF-α production between 6 and 12 h was due to THP-1 cells rather than to a further release from HUT-78 cell membranes, contact assays between THP-1 cells and membranes of HUT-78 cells were performed in the presence of cycloheximide or actinomycin D. As shown in Fig. 2, cycloheximide inhibited the increase of TNF-α production induced by membranes of HUT-78 cells, suggesting that TNF-α was newly synthesized. Similar results were obtained with actinomycin D (Fig. 2), demonstrating that transcription of TNF-α mRNA was required. The fact that neither cycloheximide nor actinomycin D abolished the basal production observed within 3 h of contact confirmed that HUT-78 cell membranes contained TNF-α.

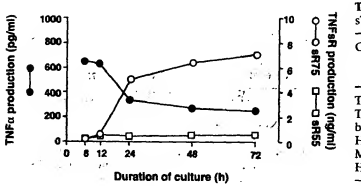

Figure 3. Production of TNF-α, sTNFR-p55 and sTNFR-p75 by THP-1 cells in contact with stimulated T cells. THP-1 cells were cultured with membranes of stimulated HUT-78 cells and TNF-α and sTNFR production was measured in the supernatant after different periods of contact.

3.2 Expression of cell-surface TNFR and release of sTNFR upon contact with membranes of stimulated HUT-78 cells

Membranes of stimulated HUT-78 cells induced the shedding of sTNFR-p75, but not sTNFR-p55 by THP-1 cells (Fig. 3). Interestingly, the appearance of sTNFR-p75 in the supernatants coincided with the disappearance of TNF-α as pointed out in Fig. 1 (Fig. 3). However, sTNFR-p75 production could not account for the decrease of TNF-α in THP-1 cell supernatants, since free and complexed TNF-α was detected by the immunoassay [28].

To find out whether sTNFR-p75 was produced by THP-1 cells or released by HUT-78 cell membranes, the expression of sTNFR-p75 on THP-1 cells and in membranes of stimulated HUT-78 cells was analyzed. THP-1 cells were cultured in the presence or absence of membranes of stimulated HUT-78 cells. Simultaneously, membranes of stimulated HUT-78 cells were incubated under similar conditions. Supernatants were collected after 48 h and THP-1 cells, HUT-78 cell membranes, or both were treated with 1 % NP40 to solubilize the cell-associated proteins. Both fractions were tested by ELISA for their content in TNFR (Table 1). Resting THP-1 cells expressed low levels of sTNFR-p75 which was not released in the supernatant. Following activation with membranes of stimulated HUT-78 cells, up to 9.8 ng/ml of sTNFR-p75 was detected in the supernatant, whereas only 0.21 ng/ml of sTNFR-p75 remained associated with the cells as detected after NP40 treatment. Since NP40 treatment did not yield detectable levels of sTNFR-p75 in the membranes of stimulated HUT-78 cells, the sTNFR-p75 detected in cultures must have been produced by THP-1 cells. This suggests that the contact with membranes of stimulated HUT-78 cells induced a strong expression of TNFR-p75 on THP-1 cells which was rapidly followed by the cleavage of the extracellular part of the receptor, *i.e.* sTNFR-p75. sTNFR-p55 was not detected under any conditions. The rapid shedding of TNFR-p75 was confirmed by binding experiments. As shown in Table 2, THP-1 cells in contact with membranes of stimulated HUT-78 cells for 48 h lost their ability to bind TNF-α.

Table 1. Expression of cell-associated TNFR-p75 and release of sTNFR-p75

Culture conditions[a]	Released sTNFR-p75[b] (ng/ml)	Cell-associated TNFR-p75[c] (ng/ml)
THP-1 cells	< 0.1	0.22
THP-1 cells + membranes of stimulated HUT-78 cells	9.8	0.21
Membranes of HUT-78 cells	< 0.1	< 0.1

a) THP-1 cells were cultured in the presence or absence of membranes of stimulated HUT-78 cells; as a control, such membranes were cultured alone and treated as THP-1 cells.
b) Supernatants were collected to measure released sTNFR-p75.
c) Cells were resuspended in medium containing 1 % NP40 to measure cell-associated TNFR-p75.

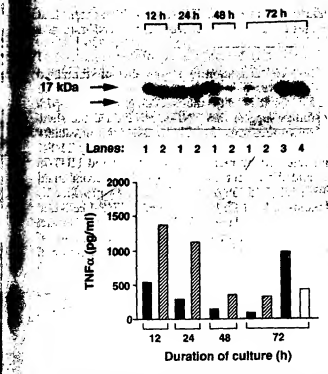

Figure 4. Degradation of TNF-α by THP-1 cells. THP-1 cells were cultured alone or with membranes of stimulated HUT-78 cells for the indicated time in the presence of ^{125}I-TNF-α. As a control, ^{125}I-TNF-α was incubated with stimulated membranes or in medium alone for 72 h. Supernatants were collected, tested for TNF-α (lower panel) and analyzed by SDS-PAGE (upper panel). THP-1 cells alone (lanes 1 and black columns); THP-1 cells + stimulated membranes (lanes 2 and dashed columns); stimulated membranes alone (lane 3 and gray column); and ^{125}I-TNF-α alone (lane 4 and open column).

3.3 Degradation of TNF-α

In all previous experiments (Figs. 1–3), a decrease of TNF-α was observed at times between 24 and 48 h suggesting that TNF-α was degraded. To test this hypothesis, ^{125}I-TNF-α was added to THP-1 cells in the presence or absence of membranes of stimulated HUT-78 cells. The supernatants were subjected to SDS-polyacrylamide gel electrophoresis and autoradiography (Fig. 4, upper panel) and measured for TNF-α content by ELISA (Fig. 4, lower panel). As shown by gel autoradiography, the 17-kDa band, *i.e.* the intact TNF-α, decreased, whereas a band of lower molecular weight appeared, suggesting time-dependent proteolytic digestion of TNF-α by THP-1 cells. The degradation of ^{125}I-TNF-α was observed when THP-1 cells were cultured in the presence or absence of T cell membranes (Fig. 4 upper panel, lanes 1). This was confirmed by ELISA (Fig. 4 lower panel), implying that activation of THP-1 cells was not required for the expression of the proteolytic activity. The proteolytic activity was associated with the cell surface and constitutive in THP-1 cells, since exogenous ^{125}I-TNF-α was not degraded by conditioned medium from resting THP-1 or membrane-activated THP-1 (data not shown).

Interestingly, when THP-1 cells were activated by PMA, autoradiography revealed similar TNF-α degradation although the production of TNF-α increased (Fig. 5). These data suggest that TNF-α production induced by

Table 2. Influence of contact with membranes of HUT-78 cells on TNF-α binding by THP-1 cells

Culture conditions[a)]	Total radioactivity (cpm)	Nonspecific binding (cpm)
THP-1 cells alone	2875 ± 126	408 ± 59
THP-1 cells + membranes of unstimulated HUT-78 cells	2399 ± 75	482 ± 67
THP-1 cells + membranes of stimulated HUT-78 cells	744 ± 100	549 ± 104

a) THP-1 cells were tested for their ability to bind exogenous ^{125}I-TNF-α after a 48-h contact with membranes of resting or stimulated HUT-78 cells.

PMA overcame the degradative capacity of THP-1 cells. To assess the specificity of TNF-α degradation, THP-1 cells were activated by membranes of stimulated HUT-78 cells in the presence of ^{125}I-IL-1β, since the latter cytokine was also induced. In contrast to TNF-α, IL-1β was not degraded by THP-1 cells (data not shown).

To characterize better the proteolytic activity responsible for TNF-α degradation, various concentrations of an inhibitor of serine proteases, PMSF, were added to THP-1 cells during the contact with membranes of stimulated HUT-78 cells. As shown in Fig. 6, up to 550 pg/ml TNF-α was

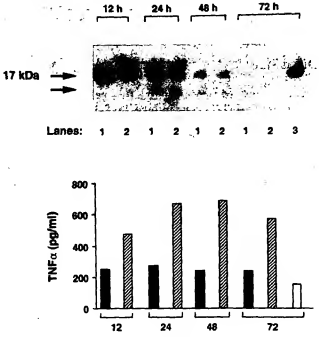

Figure 5. Degradation of TNF-α by THP-1 cells. THP-1 cells were cultured alone or with 5 ng/ml of PMA for the indicated time in the presence of ^{125}I-TNF-α. As control, ^{125}I-TNF-α was incubated in medium alone for 72 h. Supernatants were collected, tested for TNF-α (lower panel) and loaded onto SDS-polyacrylamide gel (upper panel). THP-1 cells alone (lanes 1 and black columns); THP-1 cells + membranes of stimulated HUT-78 cells (lanes 2 and dashed columns); ^{125}I-TNF-α alone (lane 3 and open column).

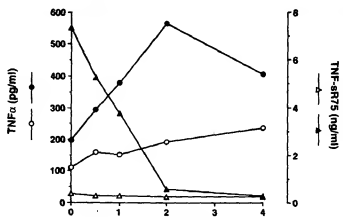

Figure 6. Dose-dependent inhibition of TNF-α degradation and sTNFR-p75 release by PMSF. THP-1 cells were incubated for 30 min in the absence or presence of PMSF, and then cultured with membranes of stimulated HUT-78 cells in the presence of 250 pg/ml ^{125}I-TNF-α (closed symbols). As control, stimulated membranes were cultured under similar conditions in the absence of THP-1 cells (open symbols). Supernatants were collected 48 h later and tested by ELISA for TNF-α (circles) and sTNFR-p75 (triangles) content.

detected after a 48-h contact between THP-1 cells and membranes of stimulated HUT-78 cells in the presence of 2 mM PMSF, whereas only 200 pg/ml was detected in the absence of PMSF. On the other hand, less than 1 ng/ml of sTNFR-p75 was detected in the presence of 2 mM PMSF, but 5 ng/ml was detected in the absence of PMSF. The dose-dependent inhibition of TNF-α proteolysis and sTNFR-p75 release by PMSF suggests that a serine protease activity is responsible for both phenomena.

4 Discussion

The key findings of this study are that cell-surface factors on stimulated HUT-78 cells induce THP-1 cells to produce TNF-α, to express TNFR-p75 and to release sTNFR-p75, and that THP-1 cells express a constitutive proteolytic activity which specifically cleaves TNF-α. Although the regulation of TNF-α activity by binding to its soluble receptors has been well described (for review, see [29]), few studies address the proteolytic degradation of soluble TNF-α. Surface peptidase activities cleaving TNF-α on U937 cells have been described [30]. The TNF-α-degrading activity of U937 cells is inhibited by serine protease inhibitors and has been attributed to both dipeptidyl aminopeptidase IV-like enzyme and tripeptidyl endopeptidase. However, these proteases generate small fragments of TNF-α, *i.e.* ≈2 kDa [30], whereas the fragments observed in our study are > 10 kDa, demonstrating that the two monocytic cell lines may express different cell-surface proteases able to cleave TNF-α. The proteolytic activity we observed was constitutively expressed by THP-1 cells (Fig. 4 and 5). However, a decrease in TNF-α production was observed after at least 12 h incubation, suggesting that the protease involved displays a low catalytic activity, despite the presence of 10% FCS. This proteolytic cleavage of TNF-α is particularly relevant to monocyte activation by contact with membranes of stimulated HUT-78 cells. Indeed, nonspecific activation of THP-1 cells by PMA induces production of TNF-α to such an extent that it overcomes the proteolytic degradation of TNF-α (Fig. 1 and 5).

Upon THP-1 cell activation by membranes of stimulated T cells, the decrease in TNF-α concentration observed after 12 h coincides with the shedding of TNFR-p75. Recent studies suggest that TNF-α can induce the shedding of its own receptors [31, 32]. To assess this possibility in our system where TNF-α is both produced by THP-1 cells and released by membranes of stimulated HUT-78 cells, we added a potent inhibitor of TNF-α, recombinant (r)sTNFR-p55hy3 fusion protein ([33], kindly provided by F. Hoffmann-La Roche), to the culture of THP-1 cells and membranes of stimulated HUT-78 cells. Two distinct experiments revealed that inhibitory concentrations of rsTNFR-p55hy3 induced only 20% and 35% inhibition of sTNFR-p75 release, suggesting that TNF-α may participate in, but is not responsible for the overall shedding of TNFR-p75 induced by the contact with membranes of stimulated HUT-78 cells. The coincidence of both TNF-α inhibitory mechanisms, *i.e.* binding to soluble receptor, forming an inactive complex, and proteolytic cleavage, was confirmed by the simultaneous inhibition of TNF-α degradation and TNFR-p75 shedding after addition of the serine protease inhibitor PMSF (Fig. 6). Whether PMSF increases the binding of TNF-α to THP-1 cells remains to be determined to exclude the possibility that the decrease of sTNFR-p75 in the presence of PMSF is due to the inhibition of receptor expression rather than the decrease of shedding. Consistent with our observations, Hwang et al. [34] have shown that PMSF inhibited sTNFR release from THP-1 cells stimulated with PMA. However, in contrast to our data, the proteolytic activity they described was associated with cell supernatants and not cell membranes. The fact that both TNF-α degradation and sTNFR-p75 shedding were simultaneously inhibited by PMSF suggests that multiple, intricate mechanisms may regulate these processes. Indeed, recent results have shown that a synthetic metalloproteinase inhibitor inhibits TNF-α processing, *i.e.* the cleavage of the 26-kDa membrane form into the 17-kDa soluble form and TNFR-p75 shedding [16]. We now show that an inhibitor of serine proteases inhibits the release of sTNFR-p75 without affecting the release of TNF-α, suggesting that these are two distinct mechanisms. Metalloproteinases have to be converted from inactive precursors to their mature and biologically active forms by specific proteolytic cleavage [35]. The serine protease activity detected here might be indirectly involved in the release of sTNFR-p75 by cleaving the metalloproteinase precursor. It might, however, be directly responsible for TNF-α degradation.

Direct cell/cell contact with membranes of stimulated HUT-78 cells induces the expression and shedding of TNFR-p75 but not of TNFR-p55 on THP-1 cells. Furthermore, as revealed by ^{125}I-TNF-α binding experiments, TNFR-p75 is rapidly shed upon contact with membranes of stimulated HUT-78 cells (Table 2). This was confirmed by ELISA measurements: most of the induced TNFR-p75 was found in the culture supernatant and not in cellular fractions of THP-1 cells activated by stimulated membranes. This demonstrates that the increase in sTNFR-p75 release does not result from the increased cleavage of pre-existing cell-associated TNFR-p75, but from an increase in

TNFR-p75 expression, immediately followed by the cleavage of its extracellular domain. These results may explain the lack of response of THP-1 cells to the large amount of TNF-α contained in membranes of stimulated HUT-78 cells [19, 20]. Furthermore, soluble TNF-α does not induce IL-1β production by THP-1 cells, *i.e.* by an autocrine mechanism as previously demonstrated [19]. This suggests that on stimulated T cell other cell-surface molecules, which remain to be identified, are involved in this phenomenon [19].

In conclusion, the present study shows that direct contact with stimulated HUT-78 cells triggers the production of TNF-α by THP-1 cells. In addition to a constitutive cell-associated proteolytic activity specifically directed at TNF-α, this signal induces a different type of regulation of TNF-α activity: the release of sTNFR-p75 which interferes with the binding of TNF-α to its target cell. It appears that serine protease(s) are responsible for both degradation of TNF-α and shedding of sTNFR-p75. This dual regulation of TNF-α activity may be of great significance in the control of inflammatory processes and immune disorders, since it may help malignant monocytic cells such as THP-1 to escape immune control.

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Thanks a lot...

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Metalloproteases and Serineproteases are Involved in the Cleavage of the Two Tumour Necrosis Factor (TNF) Receptors to Soluble Forms in the Myeloid Cell Lines U-937 and THP-1

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Björnberg F, Lantz M, Gullberg U. Metalloproteases and Serineproteases are Involved in the Cleavage of the Two Tumour Necrosis Factor (TNF) Receptors to Soluble Forms in the Myeloid Cell Lines U-937 and THP-1. Scand J Immunol 1995;42:418–424

The proteolytic processing of the two TNF receptors (TNF-R55 and TNF-R75) into soluble forms was investigated in the myeloid cell lines U-937 and THP-1. Phorbol myristate acetate (PMA) rapidly stimulated release of soluble forms of both TNF-receptors. Incubations were made with PMA and protease inhibitors directed against different target protease groups. The serineprotease inhibitors TPCK and dichloroisocoumarin and the metalloprotease inhibitor 1,10-phenanthroline reduced PMA-induced release of both soluble receptor forms with about 60–70%. Furthermore, 1,10-phenanthroline also reduced PMA-induced down-regulation of TNF-receptors in both cell lines as judged by TNF-binding to cells. Reduced down-regulation and TNF-receptor shedding by 1,10-phenanthroline was reversed by Zn^{2+}, indicating involvement of a Zn^{2+}-dependent metalloprotease. Thus, both serine proteases and metalloproteases are involved in the processing of TNF-receptors.

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INTRODUCTION

Tumor Necrosis Factor (TNF) is a pleiotropic cytokine with important functions in the inflammatory reaction [1]. TNF binds to two transmembrane receptors with molecular masses of 55 kDa (TNF-R55) and 75 kDa (TNF-R75), respectively [2]. The extracellular domains of the TNF-receptors show homology to each other, but the intracellular part of the receptors are distinct, which may indicate separate intracellular signalling pathways. Stimulation of TNF-R55 is associated with most of the biological effects exerted by TNF, such as cytotoxicity, fibroblast proliferation, antiviral activity and cytokine production [3]. Less is known about TNF-R75, but T cell proliferation [4] and inhibition of growth of primitive haematopoietic progenitor cells [5] seem to be mediated through this receptor. Transgenic mice lacking TNF-R55 are resistant to lethal dosages of bacterial endotoxin, but inoculation of the mice with the intracellular bacterial pathogen Listeria monocytogenes results in rapid death [6, 7]. Mice lacking p75 seem to be less affected, but show increased resistance to TNF-induced death and tissue necrosis [8].

TNF-receptors can undergo proteolytic cleavage with subsequent release of soluble receptor forms corresponding to the extracellular part of receptors, TNF-binding proteins (TNF-R55-BP, TNF-R75-BP). Released receptor fragments retain high affinity binding to TNF and can neutralize the effects mediated by this cytokine [9, 10]. Cleavage of TNF-receptors leads to down-regulation of TNF-binding, but internalization of intact receptor may result in down-regulation independent of receptor cleavage [11, 12]. However, shedding has been suggested as the major mechanism for down-regulation, indicating proteolytic processing at the plasma membrane [13, 14].

One or several different proteases may be responsible for the proteolytic cleavage and the protease(s) involved may prove to be tissue-specific or receptor-specific. Elucidation of mechanisms in the formation of TNF-BP by studying the protease(s) may lead to new ways of reducing the harmful manifestations of TNF. In this work we have characterized the protease(s) responsible for processing TNF-receptors in myeloid cell lines by using different protease inhibitors. The results indicate involvement of serineproteases and Zn^{2+}-dependent metalloproteases in the cleavage of both TNF-receptors.

MATERIALS AND METHODS

Reagents. Phorbol 12-myristate 13-acetate (PMA), 1,10-phenantroline and $ZnCl_2$ were from Sigma Chemical Co., St Louis, MO,

USA. The protease inhibitors antipain, aprotinin, benzamidine, bestatin, chymostatin, dichloroisocoumarin, leupeptin, pepstatin, phophoramidon, PMSF, pA-PMSF, TLCK, TPCK and EGTA were from Boehringer-Mannheim, Mannheim, Germany. Recombinant TNF-R55-BP and TNF-R75-BP were generously supplied by Dr G. Adolf, Bender & Co, Vienna, Austria and Dr H. Gallati, Hoffmann-La Roche, Basel, Switzerland, respectively.

Antibodies. Polyclonal antiserum against TNF-R55-BP was produced in rabbits as described previously [15] and has been characterized by use in immunoassays and immunoprecipitation studies [12, 15]. Monoclonal antibodies against TNF-R55-BP (TBP-1) [16] and TNF-R75-BP (Utr-4) [17] were kind gifts from Dr G. Adolf, Bender & Co, Vienna, Austria and Dr H. Gallati, Hoffman-LaRoche, Basel, Switzerland, respectively. A rat monoclonal antibody against TNF-R75 was bought from Genzyme, Cambridge, MA, USA. Peroxidase coupled goat anti-rabbit IgG was from Bio-Rad, Hercules, CA, USA and peroxidase coupled goat anti-rat IgG2b from Kemila, Sollentuna, Sweden.

Incubation of cells. The human monocyte-like cell line THP-1 and the human monoblast-like cell line U937 were maintained in RPMI supplemented with 10% fetal bovine serum (FBS). Incubations with protease inhibitors and PMA were performed at a cell concentration of $1-2 \times 10^6$ cells in 1 ml of growth medium, buffered with 10 mM HEPES, pH 7.5. At the end of the incubation specific binding of TNF to cells was determined, or cells were pelleted by centrifugation after which the supernatants were collected and frozen until analysis of TNF-R55-BP and TNF-R75-BP was performed. Cells were pre-incubated with protease inhibitors for 15 min prior to addition of PMA for stimulation for release of TNF-BP, or for down-regulation of TNF-binding.

Determination of TNF-R55-BP and TNF-R75-BP with the help of ELISA. Sandwich ELISAs for TNF-R55-BP and TNF-R75-BP were used as described previously [12, 13]. Briefly, 96-well immunoplates were coated for 3 h in a coating buffer (0.1 M $NaHCO_3$) using a monoclonal antibody (TBP-1) to TNF-R55-BP (2.5 μg/ml), or a monoclonal antibody (utr-4) to TNF-R75-BP (5 μg/ml). Plates coated with utr-4 were further incubated with 1 mg/ml BSA for 3 h at 37°C to prevent unspecific binding. Samples were loaded in duplicate and incubated overnight at 4°C. Freshly prepared dilutions of recombinant TNF-R55-BP and TNF-R75-BP were used as standards. After incubation, plates were washed three times and 100 μl of a polyclonal antiserum (diluted 1:3600) against TNF-R55-BP, or a rat monoclonal antibody against TNF-R75 (0.2 μg/ml) was added. Plates were incubated for 3 h at 20°C in an incubation buffer (0.1 M NaCl, 0.05 M NaH_2PO_4, 0.05% Tween 20) and washed three times. A peroxidase coupled goat anti-rabbit antibody (diluted 1:2500 in

Table 1. Effect of different protease inhibitors on PMA-induced release of soluble TNF-receptors

		U-937		THP-1	
Target protease	Inhibitor	TNF-R55-BP	TNF-R75-BP	TNF-R55-BP	TNF-R75-BP
	Control	50 ± 36	90 ± 17	130 ± 92	100 ± 35
	PMA (10ng/ml)	413 ± 90	600 ± 163	637 ± 393	270 ± 69
Serine	*PMA + dichloroisocoumarin (40μg/ml)	130 ± 26	233 ± 21	153 ± 42	110 ± 35
	*PMA + TPCK (100μg/ml)	137 ± 29	260 ± 55	193 ± 35	120 ± 35
	PMA + PMSF (100μg/ml)	263 ± 31	410 ± 47	400 ± 208	260 ± 35
	PMA + aprotinin (2μg/ml)	377 ± 57	430 ± 80	580 ± 398	250 ± 69
	PMA + benzamidine (1mM)	347 ± 61	427 ± 75	560 ± 364	260 ± 87
Trypsin-like serine/ cysteine	PMA + antipain (50μg/ml)	363 ± 64	440 ± 78	540 ± 312	270 ± 87
	PMA + chymostatin (100μg/ml)	293 ± 64	387 ± 40	450 ± 294	250 ± 104
	PMA + pAPMSF (50μg/ml)	325 ± 61	377 ± 30		
	PMA + TLCK (50μg/ml)	340 ± 29	370 ± 30	460 ± 242	240 ± 87
	PMA + leupeptin (2μg/ml)	353 ± 64	423 ± 52	590 ± 398	250 ± 87
Aminopeptidases	PMA + bestatin (50μg/ml)	350 ± 62	463 ± 59	520 ± 312	270 ± 87
Aspartatic	PMA + pepstatin (2μg/ml)	307 ± 57	387 ± 50	510 ± 312	220 ± 17
Metallo	*PMA + 1,10-phenanthroline (5mM)	43 ± 23	193 ± 140	220 ± 242	140 ± 104
	PMA + phosphoramidon (200μg/ml)	310 ± 0	417 ± 33	540 ± 416	250 ± 87
	PMA + EGTA (5mM)	236 ± 52	434 ± 113	440 ± 277	190 ± 52

Cells (2×10^6) were incubated in 1 ml of complete medium at 37°C for 2 h with PMA alone or in combination with protease inhibitors. Cells were pre-incubated for 15 min with protease inhibitors prior to addition of PMA. Cell supernatants were collected and analysed for TNF-R55-BP and TNF-R75-BP as described in Materials and Methods. Values are mean from three separate experiments (± S.D.), pg/ml.

*Protease inhibitors with the most profound effect on the PMA-induced production of soluble TNF-receptors.

incubation buffer) or a peroxidase coupled goat anti-rat IgG 2b (diluted 1:2500 in incubation buffer) was allowed to bind during incubation for 1 h at 20°C . After washing, tetrametyl benzidine (TSI, Milford, MA, USA) was added as substrate and absorbance was measured at 660 nm, or after addition of 1 M H_2SO_4, at 450 nm in a Titertek multiscan ELISA plate reader. Values were calculated from a standard curve based on freshly prepared dilutions of TNF-R55-BP and TNF-R75-BP. The detection limit for the TNF-R55-BP assay was 0.03 ng/ml and 0.08 ng/ml for the TNF-R75-BP assay.

Determination of specific binding of TNF to cells. Iodination of TNF was carried out at 4°C with ^{125}I-NaI (Amersham, Amersham, UK) using Iodobeads® (Pierce, Oud Beijerland, the Netherlands) according to the manufacturer's instruction. Specific binding of iodinated TNF to cells was determined as described previously [12, 18]. Briefly, 4×10^6 cells were incubated with ^{125}I-TNF in 200 μl of binding buffer consisting of Dulbecco's phosphate-buffered saline (PBS), pH 7.4, supplemented with 1% bovine serum albumin (BSA) under gentle rotation for 2 h, after which the cells were washed twice with cold binding buffer and remaining cell-associated radioactivity was determined by use of a gamma counter. Non-specific binding of TNF was determined in the presence of 100-fold excess of unlabelled TNF and was 5–15% of total binding.

RESULTS

Inhibitors of serineproteases and metalloproteases reduces PMA-induced release of soluble TNF-receptor forms

PMA induces the proteolytic processing of the TNF receptors and the subsequent release of soluble receptor forms from the cells. In order to characterize the proteases involved, cells were treated with different protease inhibitors and incubated with PMA followed by detection of soluble TNF-receptor forms. The inhibitors were directed against five different target protease groups, serine proteases, trypsin-like serine/cysteineproteases, aminopeptidases, aspartatic proteases, and metalloproteases. Table 1 shows that the serineprotease inhibitors dichloroisocoumarin and TPCK strongly reduced the release of TNF-R55-BP and TNF-R75-BP from PMA-stimulated U937 and THP-1 cells with 60–70% in both cell lines. Other serine protease inhibitors tested proved to be less active or inactive in reducing receptor release from the PMA-stimulated cell lines. Likewise inhibitors of trypsin-like proteases, aminopeptidases, and aspartatic proteases had negligible effects on release of soluble TNF-receptor forms.

Among inhibitors of metalloproteases, 1,10-phenanthroline, phosphoramidon, and EGTA were investigated. 1,10-phenanthroline and EGTA inhibit metal-dependent proteases by acting as chelators of divalent cations such as Mg^{2+} and Zn^{2+}. Phosphoramidon especially inhibits metallo-endopeptidases of bacterial origin [19]. As shown in Table 1, both EGTA and 1,10-phenanthroline reduced the PMA-induced release of soluble receptor with approximately 30% and 70%, respectively. A dose-dependent inhibition of TNF-receptor release by dichloroisocoumarin, TPCK and 1,10-phenanthroline is shown in Fig. 1. Concentrations higher than 40 μg/ml of dichloroisocoumarin, 100 μg/ml of TPCK, and 5 mmol/l of 1,10-phenanthroline were unable to be used due to toxicity to the cells, as measured by Trypan-blue exclusion. These results indicate the involvement of enzymes with characteristics of serineproteases and metalloproteases in the proteolytic processing of TNF-receptors.

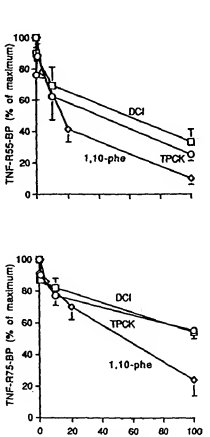

Fig. 1. Dose-dependent inhibition of PMA-induced release of TNF-R55-BP and TNF-R75-BP by TPCK, dichloroisocoumarin and 1,10-phenanthroline. U-937 cells (2×10^6/ml) were incubated at 37°C with the protease inhibitors TPCK, dichloroisocoumarin and 1,10-phenanthroline at indicated concentrations for 15 min, after which PMA (10 ng/ml) was added and cells were incubated further for 2 h. Cell supernatants were collected and analysed for TNF-R55-BP and TNF-R75-BP by ELISA. Results are normalized and expressed as the percentage of release induced by PMA alone. Values shown are mean values from three experiments separate from those presented in Table 1. Bars represent S.D. and bars not shown fall within the limit of the symbols.

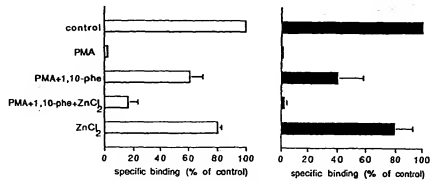

Fig. 2. Inhibition of PMA-induced down-regulation of TNF-receptors by 1,10-phenanthroline and reversal of the inhibition by Zn^{2+}. U 937 (□) and THP-1 cells (■) (1×10^6/ml) were pre-incubated at 37°C for 15 min with 5 mM 1,10-phenanthroline and $ZnCl_2$ (U937, 1 mM; THP-1, 2 mM) as indicated. Following pre-incubation, 10 ng/ml of PMA was added, after which continued incubation for 30 min followed. Control incubations were performed without additions. Results are normalized and percentage of specific binding of TNF as compared with control cells is shown. Specific binding to control cells was 6900(±690 S.D.) cpm (U-937) and 2700(±1700 S.D.) cpm (THP-1). Bars represent range from three separate experiments and bars not shown fall within the limit of the symbols.

PMA-induced down-regulation of TNF-binding to cells depends on metalloprotease activity

Activation of cells with PMA induces a rapid down-regulation of TNF-binding capacity of the cells [20]. Down-regulation may result from proteolytic cleavage of the receptors with subsequent release of soluble receptor forms, or it may occur due to internalization of intact receptor, both events leading to decreased ligand binding. Some data indicate that cleavage and internalization can be two separate processes [11, 12]. As described above, the production of soluble receptor forms was found to be strongly reduced by 1,10-phenanthroline, an inhibitor of metalloproteases. Therefore, we wanted to investigate whether also down-regulation of TNF-binding, that hypothetically may be due to receptor internalization, was inhibited by this agent. Figure 2 shows that incubation with PMA, that resulted in down-regulation of the TNF-binding to U-937 and THP-1 cells, was inhibited by 1,10-phenanthroline. Thus the PMA-induced down-regulation seems to involve metalloproteases and cleavage of TNF-receptors at the cell surface.

The inhibitory effects of 1,10-phenanthroline on soluble TNF-receptor release and receptor down-regulation depend on Zn^{2+}

Metalloproteases depend on divalent cations to be enzymatically functional. 1,10-phenanthroline is considered to function as a chelator of cations, with its highest affinity for Zn^{2+}. To further characterize the action of this protease inhibitor it was investigated whether its effect on soluble receptor release and receptor down-regulation was dependent on the concentration of Zn^{2+}. Fig 2 shows that Zn^{2+}, which alone resulted in only a small down-regulation of TNF-binding, strongly reversed the action of 1,10-phenanthroline on PMA-induced down-regulation in both U-937 and THP-1 cells. A molar

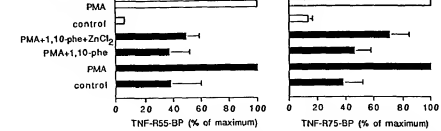

Fig. 3. The inhibitory effect of 1,10-phenanthroline on PMA-induced release of TNF-R55-BP and TNF-R75-BP is partially reversed by Zn^{2+}. U 937 (□) and THP-1 cells (■) (2×10^6/ml) were pre-incubated at 37°C for 15 min with 5 mM 1,10-phenanthroline and $ZnCl_2$ (U937, 1 mM; THP-1, 2 mM) as indicated. Following pre-incubation, 10 ng/ml of PMA was added, after which continued incubation for 2 h followed. Control incubation were performed without addition of PMA. Cell supernatants were collected and analysed for TNF-R55-BP and TNF-R75-BP. Results are normalized and expressed as the percentage of the release induced by PMA alone, and represent mean values from three separate experiments. Bars represent range and bars not shown fall within the limit of the symbols.

excess of Zn^{2+}, which might have reversed the action of 1,10-phenanthroline even further, was not possible to use due to toxic effects on the cells. Similar to down-regulation of TNF-binding, the inhibition of PMA-induced release of soluble TNF-R55-BP and TNF-R75-BP by 1,10-phenanthroline was partially reversed by the simultaneous addition of Zn^{2+} (Fig. 3). The reversal of the effect of 1,10-phenanthroline by Zn^{2+} seems to be less pronounced on the release of soluble receptor forms, as compared with down-regulation of TNF-binding. However, the assays may, in this respect, be difficult to compare, as down-regulation is determined after 30 min of incubation, while the release of soluble TNF-receptors is allowed to continue for 2 h. It cannot be excluded that, in addition to effects on receptor cleavage, effects on receptor synthesis may be evident during the 2 h incubation. However, together these results further support the notion of a role for Zn^{2+}-dependent metalloproteases in PMA-induced proteolytic processing and down-regulation of TNF-receptors in U-937 and THP-1 cells.

DISCUSSION

In the present study we demonstrate that certain serine protease inhibitors, and in particular the metalloprotease inhibitor 1,10-phenanthroline, inhibit PMA-induced release of soluble forms of the two TNF-receptors in the myeloid cell lines U-937 and THP-1. The inhibition of the proteolytic processing by inhibitors directed both against serine and metalloproteases suggests that at least two distinct proteases are involved in the cleavage of TNF-receptors. The sensitivity of TNF-receptor shedding to inhibitors of serineproteases (TPCK and dichloroisocoumarine) is in agreement with previously reported results [21]. Our present finding that EGTA and 1,10-phenanthroline reduce the cleavage of TNF-receptors also suggests that metalloproteases are involved in the processing TNF-R55 and TNF-R75. Among the metalloprotease inhibitors tested, 1,10-phenanthroline showed a strong effect while the inhibition exerted by EGTA was moderate or low. As 1,10-phenanthroline is a strong chelator of metal ions, and in particular Zn^{2+}, this suggests the involvement of a Zn^{2+}-dependent metalloprotease for enzymatic activity. This notion is concordant with our finding that the inhibitory effect of 1,10-phenanthroline was reversible by addition of Zn^{2+}.

Cleavage and shedding of cell surface receptors may be a mechanism for down-regulation of ligand binding. However, down-regulation can occur in the absence of cleavage, as the result of internalization of the intact receptor. Our finding that not only PMA-induced release of soluble receptor forms, but also down-regulation of TNF-binding, was inhibited by 1,10-phenanthroline in a Zn^{2+}-dependent manner, indicate that proteolytic cleavage at the cell surface is responsible for PMA-induced down-regulation of TNF-binding.

It is important to emphasise that PMA, used to induce shedding of the TNF-receptors can also induce transcription of TNF-R mRNA and following translation into protein [22, 23]. It may be difficult, therefore, to distinguish between inhibitory effects on protein synthesis and on receptor cleavage/shedding. However, our finding that the short-term PMA-induced down-regulation of TNF-R expression on the cell surface is also reversed by 1,10-phenantroline, demonstrates that the protease inhibitor indeed affects late proteolytic events.

A wide variety of transmembrane proteins, including cell adhesion molecules, membrane-anchored growth factor precursors, growth factor and cytokine receptors are proteolytically cleaved in a regulated process to form soluble counterparts of the membrane proteins [24]. The identities of the proteases involved in these processes are mostly unknown, but the low affinity nerve growth factor (NGF)-receptor, which is a member of the TNF-receptor superfamily [3, 25], displays a 1,10-phenanthroline-sensitive proteolytic processing in Schwann cells [26]. The cleavage of NGF-receptor was insensitive to inhibitors of serineproteases. A metalloprotease, sensitive to 1,10-phenanthroline, has been suggested to participate in the cleavage of CD16, CD43 and CD44 in neutrophil granulocytes [27] and similar to our present results, this processing was also sensitive to some inhibitors of serineproteases. It has been postulated that two separate mechanisms are responsible for cleavage of CD43 in neutrophils: a PMA-induced CD43′ase and a second serine-protease [28]. The putative CD43′ase was, however, insensitive to 1,10-phenantroline, thus making it distinct from the metalloprotease characterised in this study. A transmembrane form of the cytokine TNF is proteolytically processed into soluble forms at the cell-surface involving Zn^{2+}-dependent matrix metalloproteases [29–31]. In addition, it has recently been reported that a metallo-protease inhibitor affecting the cleavage of TNF also blocks the shedding of TNF-R80 from T-lymphocytes [32]. Some matrix metalloproteases are proteolytically activated by serineproteases. For example, collagenase, present in a catalytically inactive form in neutrophils, can be activated by proteolytic processing by serine proteases [33]. In analogy, a 1,10-phenanthroline-sensitive metalloprotease, cleaving TNF-receptor may hypothetically depend on serineproteases for catalytic activation in cells utilized in this study. Although not demonstrated, such a serine-metalloprotease cascade could explain the reduction of soluble TNF-receptor release by inhibitors of both metalloproteases and serine-proteases in U-937 and THP-1 cells.

The present results indicate similar sensitivity of processing of the two TNF-receptors to protease inhibitors. This finding suggests that common proteases may be responsible for cleavage of both TNF-receptors in U937 and THP-1 cells. It is important, however, to emphasize that distinct and tissue-specific proteolytic mechanisms for transmembrane proteins may occur. For example, we have previously shown that NH_4Cl and monensin, two agents known to increase lysosomal pH, reduced PMA-induced release of TNF-receptors in myeloid cell lines while TNF-receptor

processing in neutrophils was unaffected by NH_4Cl and monensin (data not shown). *In vivo*, different carboxy-terminals of purified TNF-BP have been reported [34], giving further support for distinct proteolytic mechanisms. In conclusion, the present investigation demonstrates that PMA-induced proteolytic cleavage of soluble TNF-receptors and down-regulation of TNF-binding in myeloid cell lines depend on proteases belonging to the families of serine-proteases and metalloproteases. Identification of the proteases responsible for TNF-receptor processing could prove to be valuable in the development of pharmacological drugs to modulate the effects of TNF *in vivo*.

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